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(54) Title: RECOMBINANT PROTEINS HAVING MULTIPLE DISULFIDE BONDS AND THIOL-SUBSTITUTED CONJUGATES THEREOF

(57) Abstract

The present invention relates to recombinant antigen binding proteins having multiple disulfide bonds useful for the preparation of immunoconjugates. In particular, this invention relates to recombinant antibodies comprising an IgG_3 hinge region and lacking a CH2 constant domain. These mutated antibodies are used to bind a diagnostic or therapeutic agent through one or more reduced disulfide bonds in the antibody hinge region. Thus, the invention contemplates the use of such immunoconjugates in diagnosis and therapy.

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RECOMBINANT PROTEINS HAVING MULTIPLE DISULFIDE BONDS AND THIOL-SUBSTITUTED CONJUGATES THEREOF

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention is directed to recombinant antigen binding proteins having multiple disulfide bonds useful for the preparation of diagnostic and therapeutic conjugates. In particular, this invention is directed to recombinant antibodies comprising an IgG_3 hinge region and lacking a CH2 constant domain. This invention also is directed to immunoconjugates comprising such a recombinant antibody which is covalently bound to a diagnostic or therapeutic agent through one or more reduced disulfide bonds in the antibody hinge region. This invention is further directed to methods for preparing such immunoconjugates. This invention also is directed to methods of diagnosis and therapy using such immunoconjugates.

2. Related Art

Monoclonal antibodies can be conjugated to a variety of haptens to form immunoconjugates for use in diagnosis and therapy. These agents include chelates, which allow the immunoconjugate to form a stable bond with radioisotopes, and cytotoxic agents such as toxins and chemotherapy drugs. For example, cytotoxic agents that normally would be too toxic to patients when administered in a systemic fashion can be coupled to anti-cancer antibodies in such a manner that their toxic effects become directed only to the tumor cells bearing the target antigens. The diagnostic or therapeutic efficacy of immunoconjugates depends upon several factors. Among these factors, the molar ratio of the diagnostic or therapeutic agent to antibody and the antibody binding activity of the immunoconjugate are of major concern.

Researchers have found that the maximum number of diagnostic or therapeutic agents that can be directly linked to an antibody is limited by the number of modifiable sites on the antibody molecule and the loss of

immunoreactivity of the antibody. For example, Kulkarni *et al.*, *Cancer Research* 41:2700-2706 (1981), have reported that there is a limit to the number of drug molecules that can be incorporated into an antibody without significantly decreasing antigen-binding activity. Kulkarni *et al.*, found that the highest incorporation obtained for methotrexate was about ten methotrexate molecules per molecule of antibody, and that attempts to increase the drug-antibody molar ratio over about ten decreased the yield of immunoconjugate and damaged antibody activity. Kanellos *et al.*, *JNCI* 75:319-329 (1985), have reported similar results.

Accordingly, the attachment of haptens, particularly in a multiple manner, at nonspecific sites on monoclonal antibodies (Mabs) can lead to a reduction, or a complete loss, of the ability of the Mab to bind antigen. Typically, a lysine residue is used for nonspecific substitutions due to the ease of performing chemical substitutions at the free amino group. However, there is a risk of attaching a hapten to a lysine residue in the antigen binding region of a Mab with a consequent loss of antigen-binding ability.

Methods have been developed to alleviate the risk of binding haptens to the antigen-binding domains of Mabs. One approach is to attach haptens via Mab carbohydrate groups, which normally are located in the constant (CH2 domain) and distant from antigen-binding domains. According to this technique, vicinal diols of carbohydrate are oxidized to aldehydes, and the amino group of a hapten is coupled to the aldehydes to form a Schiff base. Optionally, the Schiff base can be reduced to an amino bond.

For example, Shih *et al.*, *Int. J. Cancer* 41:832-839 (1988), have described a site-specific linking method in which methotrexate was linked to the carbohydrate moiety in the constant, or "Fc," region of an antibody via amino-dextran, resulting in an immunoconjugate with high substitution ratio and retention of immunoreactivity.

More recently, Shih *et al.*, *Int. J. Cancer* 46:1101-1106 (1990), demonstrated the efficacy of an immunoconjugate comprising 5-fluorouridine conjugated via amino-dextran to the carbohydrate moiety in the Fc region of a monoclonal antibody. In both studies, Shih *et al.* found that the immunoconjugate contained approximately 30-50 molecules of drug per molecule of immunoglobulin. Thus, indirect conjugation of a diagnostic or therapeutic agent to a carbohydrate moiety in the Fc region of an antibody provides a means to obtain immunoconjugates with functional antigen binding activity and a high substitution level.

However, a disadvantage of using the carbohydrate moiety in the Fc region as an attachment site is that the entire antibody is required for the immunoconjugate. The use of antibody fragments, particularly Fab, Fab' and $F(ab')_2$ provide an advantage over the use of an entire antibody because such fragments are better able to diffuse out of capillaries and into target tissues. For example, see Brown, "Clinical Use of Monoclonal Antibodies," in BIOTECHNOLOGY AND PHARMACY, Pezzuto *et al.*, eds. Chapman & Hall, pp.227-249 (1993).

In an alternative approach, haptens are attached to Mabs via thiol groups that have been generated by reducing Mab disulfide bonds. Although a significant proportion of inter-chain disulfide bonds may be reduced, the Mab heavy chains and light chains are held together via electrostatic and hydrophobic interactions.

The attachment of haptens via reduced disulfide bonds offers several advantages. Since disulfide bonds are not located near antigen-binding domains, the reduction of the disulfide bonds will not interfere with antigen binding by the hapten-antibody conjugate. Unlike carbohydrate, disulfide (or thiol) groups are universally present in antibodies including humanized Mab constructs prepared by bacterial fermentation. Furthermore, in contrast to thiolated proteins prepared by a thiolation reaction, sulfhydryl-containing proteins prepared from

intrinsic Mab disulfide bonds do not produce aggregated Mab side-products.

For *in vivo* diagnostic or for therapeutic applications, there are certain advantages in using hapten-bearing $F(ab)_2/F(ab')_2$ fragments of Mabs, as opposed to intact Mabs. For example, $F(ab)_2/F(ab')_2$ fragments are taken up by the liver at a much lower rate than intact Mabs due to the lack of Fc domains. In addition, blood retention times are shorter with $F(ab)_2/F(ab')_2$ fragments, compared with intact Mabs. These characteristics are particularly useful for decreasing radiotoxicity in radioimmunodetection and radioimmunotherapy methods. Moreover, one can obtain a more even distribution of haptens using $F(ab)_2/F(ab')_2$ fragments due to the greater penetration into target sites of the smaller molecules.

Haptens also can be bound to Fab/Fab' fragments which have even shorter half-lives in circulation than $F(ab)_2/F(ab')_2$ fragments. These smaller molecules, however, are characterized by a lower rate of uptake in target tissue and by shorter retention time in target tissue due to the monovalent character of the fragments. Unlike the smaller monovalent fragments, $F(ab)_2/F(ab')_2$ fragments retain the divalent character of intact Mabs that provides the high level of antigen binding capacity. Accordingly, Fab/Fab' fragments are less suitable as therapeutic agents, compared with $F(ab)_2/F(ab')_2$ fragments, because the uptake of a therapeutic agent in a target is a critical determinant of therapeutic efficacy.

Although there are many advantages to using $F(ab)_2/F(ab')_2$ fragments in diagnosis and therapy, a disadvantage is that the fragments are less amenable to successful non-site-specific hapten attachment compared with intact Mab. This is so because the likelihood that a hapten will bind with a lysine residue in a antigen-binding site is increased in the smaller molecules.

Moreover, th fragments typically lack carbohydrate residues because the CH2 constant region is destroyed during enzymatic cleavage of the intact Mab. Finally, reduction of $F(ab)_2/F(ab')_2$ fragments for thiol attachment can cleave the divalent fragments to produce their monovalent counterparts. The Fab/Fab' fragments lack constant domains to promote reassociation to the divalent form.

Thus, a need exists for an antigen-binding molecule that combines the antigen-binding capacity and hapten-binding capacity of intact antibodies with the rapid clearance and high tissue penetrability of antibody fragments.

SUMMARY OF THE INVENTION

Accordingly, it is an object of the present invention to provide a mutated antibody comprising a heavy chain that contains a hinge region with multiple disulfide bonds, but lacks a CH2 domain.

Another object of this invention is to provide methods for selectively targeting diagnostic and therapeutic agents to tumor cells or to infectious agents, while avoiding major toxic side effects to normal organs.

These and other objects are achieved, in accordance with one embodiment of the present invention by the provision of a mutated antibody comprising a heavy chain having (a) a variable region, (b) a CH1 domain, (c) a hinge region having three or more disulfide bonds, and (d) a CH3 domain, and lacking a CH2 domain.

The present invention also is directed to such mutated antibodies wherein the hinge region is a human IgG_3 hinge region.

The present invention is further directed to mutated antibodies having a variable region that binds with an antigen associated with a tumor or an infectious agent.

The present invention also contemplates mutated antibodies that further comprise a light chain comprising

a carbohydrate moiety attached at about amino acid 18 in the variable region of the light chain.

The present invention also is directed to immunoconjugates comprising a mutated antibody and at least one diagnostic or therapeutic agent. Suitable diagnostic agents include a radioactive label, photoactive agent or dye, florescent label, enzyme label, bioluminescent label, chemiluminescent label, colloidal gold and paramagnetic ion. Suitable therapeutic agents are selected from the group consisting of radioisotope, boron addend, immunomodulator, toxin, photoactive agent or dye, cancer chemotherapeutic drug, antiviral drug, antifungal drug, antibacterial drug and antiprotozoal drug.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a representation of the model mutated antibody described herein.

Figure 2 shows high-pressure liquid chromatography traces of an antibody-streptavidin conjugate prepared by two different methods.

Figure 3 shows a map of the plasmid, LL2-14pG1g.

Figure 4 shows maps of the vectors, CHpBSK and ΔC2h3pBSK.

Figure 5 shows a map of the plasmid, hMN-14ΔC2h3pG1g.

Figure 6 shows a map of the plasmid, hMN-14pdHL2.

Figure 7 shows a map of the plasmid, hMN-14ΔC2h3pdHL2.

Figure 8 presents the structure of calicheamicin, where R' is rhamnose, R'' is an aminosugar, and R''' is a CH_3-S group that forms a trisulfide bond with sulfur. The figure also illustrates how a calicheamicin immunoconjugate would cleave DNA *in vivo*.

DETAILED DESCRIPTION

1. Overview

To overcome the problem of hapten attachment, a novel construct was designed in which the IgG_1 hinge region and the CH2 domain of a humanized antibody were deleted and were replaced with a human IgG_3 hinge region. The

humanized antibody, hMN-14 ("hIMMU-14"), was used as a prototype. The hMN-14 antibody is an anti-CEA antibody having the human IgG_1/κ isotype. Shevitz *et al.*, *J. Nucl. Med.* 35:112 (1994). In the engineered antibody, hIMMU-14-ΔCH2-IgG_3, the IgG_1 hinge region is replaced with an IgG_3 hinge region. In addition, the IgG_1 CH2 domain is deleted. The resultant structure of the hIMMU-14-ΔCH2-IgG_3 heavy chain is: [hMN14 VH]-[CH1 (IgG_1)]-[hinge(IgG_3)]-[CH3(IgG_1)]. Depending on the application, the VK domain of hMN-14 may be engineered with an N-linked glycosylation site.

This construct retains the advantageous properties of divalent Mabs and fragments, but lacks the disadvantages of each form. Specifically, the lack of CH2 domain leads to reduced hepatic uptake and liver toxicity, compared with intact Mabs. The greater size of the new construct results in reduced renal uptake and kidney toxicity, compared with $F(ab)_2/F(ab')_2$ fragments. This is so because the new construct has a molecular weight above the level where kidney sequestration is observed. However, the extended disulfide hinge region together with the CH1 region inhibits cleavage of the mutated antibody to monovalent fragments in blood.

In addition, the extended hinge region with 11 sulfhydryl groups provides for the multiple substitution of haptens via thiol groups, unlike $F(ab)_2/F(ab')_2$ fragments. Multi-drug loading with minimal impingement on antibody function is an important feature of the new construct. Figure 1 illustrates the model mutated antibody described herein with the extended hinge region.

Moreover, the new construct associates to a dimeric fragment from monomeric units in an efficient manner due to the presence of multiple disulfide bonds and the retention of a significant portion of the constant region. This feature is not only important for the efficient production of the constructs *in vitro*, but also is significant because mild reduction for hapten attachment will not cause cleavage to monomeric subunits.

Moreover, the new construct has greater penetrative capability than the larger intact Mabs due to the smaller size of the new construct.

Finally, a VK-appended carbohydrate moiety at an engineered glycosylation site can serve as an additional conjugation site to increase the loading capacity or to conjugate haptens that are different from those conjugated at the hinge region.

2. Definitions

In the description that follows, a number of terms are utilized extensively. Definitions are herein provided to facilitate understanding of the invention.

Mutated Antibody. As used herein, a mutated antibody comprises a heavy chain that contains hinge region having three or more disulfide bonds, but lacks a CH2 constant domain.

Diagnostic or Therapeutic Agent. As used herein, a diagnostic or therapeutic agent is a molecule or atom which is conjugated to an antibody moiety to produce an immunoconjugate that is useful for diagnosis and for therapy. Examples of diagnostic or therapeutic agents include drugs, toxins, immunomodulators, chelators, boron compounds, photoactive agents or dyes, radioisotopes, fluorescent agents, paramagnetic ions or molecules and marker moieties.

Immunoconjugate. As used herein, an immunoconjugate is a molecule comprising a mutated antibody and a diagnostic or therapeutic agent. An immunoconjugate retains the immunoreactivity of the mutated antibody, *i.e.*, the antibody moiety has roughly the same, or only slightly reduced, ability to bind the antigen after conjugation as before conjugation.

Structural gene. A DNA sequence that is transcribed into messenger RNA (mRNA) which is then translated into a sequence of amino acids characteristic of a specific polypeptide.

Prom ter. A DNA sequence which directs the transcription of a structural gene to produce mRNA.

Typically, a promoter is located in the 5' region of a gene, proximal to the start codon of a structural gene. If a promoter is an inducible promoter, then the rate of transcription increases in response to an inducing agent. In contrast, the rate of transcription is not regulated by an inducing agent if the promoter is a constitutive promoter.

Enhancer. A promoter element. An enhancer can increase the efficiency with which a particular gene is transcribed into mRNA irrespective of the distance or orientation of the enhancer relative to the start site of transcription.

Complementary DNA (cDNA). Complementary DNA is a single-stranded DNA molecule that is formed from an mRNA template by the enzyme reverse transcriptase. Typically, a primer complementary to portions of mRNA is employed for the initiation of reverse transcription. Those skilled in the art also use the term "cDNA" to refer to a double-stranded DNA molecule consisting of such a single-stranded DNA molecule and its complement.

Expression. Expression is the process by which a polypeptide is produced from a structural gene. The process involves transcription of the gene into mRNA and the translation of such mRNA into polypeptide(s).

Cloning vector. A DNA molecule, such as a plasmid, cosmid, phagemid, or bacteriophage, which has the capability of replicating autonomously in a host cell and which is used to transform cells for gene manipulation. Cloning vectors typically contain one or a small number of restriction endonuclease recognition sites at which foreign DNA sequences may be inserted in a determinable fashion without loss of an essential biological function of the vector, as well as a marker gene which is suitable for use in the identification and selection of cells transformed with the cloning vector. Marker genes typically include genes that provide tetracycline resistance or ampicillin resistance.

Expression vector. A DNA molecule comprising a cloned structural gene encoding a foreign protein which provides the expression of the foreign protein in a recombinant host. Typically, the expression of the cloned gene is placed under the control of (*i.e.*, operably linked to) certain regulatory sequences such as promoter and enhancer sequences. Promoter sequences may be either constitutive or inducible.

Recombinant Host. A recombinant host may be any prokaryotic or eukaryotic cell which contains either a cloning vector or expression vector. This term is also meant to include those prokaryotic or eukaryotic cells that have been genetically engineered to contain the cloned gene(s) in the chromosome or genome of the host cell. For examples of suitable hosts, see Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL, Second Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989).

A tumor associated antigen is a protein normally not expressed, or expressed at very low levels, by a normal counterpart. Examples of tumor associated antigens include α-fetoprotein and carcinoembryonic antigen (CEA). Many other illustrations of tumor associated antigens are known to those of skill in the art. See, for example, Urban *et al.*, *Ann. Rev. Immunol. 10*: 617 (1992).

As used herein, an **infectious agent** denotes both microbes and parasites. A "microbe" includes viruses, bacteria, rickettsia, mycoplasma, protozoa, fungi and like microorganisms. A "parasite" denotes infectious, generally microscopic or very small multicellular invertebrates, or ova or juvenile forms thereof, which are susceptible to antibody-induced clearance or lytic or phagocytic destruction, such as malarial parasites, spirochetes, and the like.

Humanized antibodies are recombinant proteins in which murine complementary determining regions of monoclonal antibodies have been transferred from heavy

and light variable chains of the murine immunoglobulin into a human variable domain.

As used herein, the term **antibody component** includes both an entire antibody and an antibody fragment.

3. Methods for Preparing a Mutated Antibody That Contains Multiple Disulfide Bonds in the Hinge Region

A. Production of Rodent Monoclonal Antibodies, Humanized Antibodies, Primate Antibodies and Human Antibodies

A mutated antibody of the present invention may be derived from a rodent monoclonal antibody. Rodent monoclonal antibodies to specific antigens may be obtained by methods known to those skilled in the art. *See*, for example, Kohler and Milstein, *Nature 256*: 495 (1975), and Coligan *et al.* (eds.), CURRENT PROTOCOLS IN IMMUNOLOGY, VOL. 1, pages 2.5.1-2.6.7 (John Wiley & Sons 1991) [hereinafter "Coligan"]. Briefly, monoclonal antibodies can be obtained by injecting mice with a composition comprising an antigen, verifying the presence of antibody production by removing a serum sample, removing the spleen to obtain B-lymphocytes, fusing the B-lymphocytes with myeloma cells to produce hybridomas, cloning the hybridomas, selecting positive clones which produce antibodies to the antigen, culturing the clones that produce antibodies to the antigen, and isolating the antibodies from the hybridoma cultures.

Mabs can be isolated and purified from hybridoma cultures by a variety of well-established techniques. Such isolation techniques include affinity chromatography with Protein-A Sepharose, size-exclusion chromatography, and ion-exchange chromatography. See, for example, Coligan at pages 2.7.1-2.7.12 and pages 2.9.1-2.9.3. Also, see Baines *et al.*, "Purification of Immunoglobulin G (IgG)," in METHODS IN MOLECULAR BIOLOGY, VOL. 10, pages 79-104 (The Humana Press, Inc. 1992).

A wide variety of monoclonal antibodies against tumor associated antigens or infectious agents have been developed. See, for example, Goldenberg *et al.*,

international application publication No. WO 91/11465 (1991), Hansen *et al.*, international application publication No. WO 93/23062, and Goldenberg, international application publication No. WO 94/04702 (1994), each of which is incorporated by reference in its entirety.

Furthermore, such antibodies are readily available from commercial sources. For example, rodent monoclonal antibodies that bind with adenocarcinoma-associated antigen (Cat. No. 121730), human chorionic gonadotropin (Cat. No. 230740), carcinoembryonic antigen (Cat. Nos. 215920 and 215922), human alpha-fetoprotein (Cat. No. 341646), and the like can be obtained from Calbiochem-Novabiochem Corp. (San Diego, CA). Moreover, rodent monoclonal antibodies that bind with antigenic determinants of infectious agents such as *Escherichia coli* (HB 8178), *Legionella pneumophila* (CRL 1770), *Schistosoma mansoni* (HB 8088), *Streptococcus*, Group A (HB 9696), *Treponema pallidum* (HB 8134), hepatitis B (CRL 8017), herpes simplex (HB 8181), human immunodeficiency virus (HB 9101), among others, can be obtained from American Type Culture Collection (Rockville, MD). Furthermore, murine monoclonal antibodies against merozoites and sporozoites of *Plasmodium falciparum* can be prepared as described by Goldenberg, U.S. patent No. 5,332,567 (1994), which is incorporated by reference.

A mutated antibody of the present invention may also be derived from a subhuman primate antibody. General techniques for raising therapeutically useful antibodies in baboons may be found, for example, in Goldenberg *et al.*, international patent publication No. WO 91/11465 (1991), and in Losman *et al.*, *Int. J. Cancer 46*: 310 (1990), which is incorporated by reference.

Alternatively, a mutated antibody may be derived from a "humanized" monoclonal antibody. Humanized monoclonal antibodies are produced by transferring mouse complementary determining regions from heavy and light variable chains of the mouse immunoglobulin into a human

variable domain, and then, substituting human residues in the framework regions of the murine counterparts. The use of antibody components derived from humanized monoclonal antibodies obviates potential problems associated with the immunogenicity of murine constant regions. General techniques for cloning murine immunoglobulin variable domains are described, for example, by the publication of Orlandi *et al.*, *Proc. Nat'l Acad. Sci. USA 86*: 3833 (1989), which is incorporated by reference in its entirety. Techniques for producing humanized Mabs are described, for example, by Jones *et al.*, *Nature 321*: 522 (1986), Riechmann *et al.*, *Nature 332*: 323 (1988), Verhoeyen *et al.*, *Science 239*: 1534 (1988), Carter *et al.*, *Proc. Nat'l Acad. Sci. USA 89*: 4285 (1992), Sandhu, *Crit. Rev. Biotech. 12*: 437 (1992), and Singer *et al.*, *J. Immun. 150*: 2844 (1993), each of which is hereby incorporated by reference.

As an alternative, a mutated antibody of the present invention may be derived from human antibody fragments isolated from a combinatorial immunoglobulin library. See, for example, Barbas *et al.*, *METHODS: A Companion to Methods in Enzymology 2*: 119 (1991), and Winter *et al.*, *Ann. Rev. Immunol. 12*: 433 (1994), which are incorporated by reference. Cloning and expression vectors that are useful for producing a human immunoglobulin phage library can be obtained, for example, from STRATAGENE Cloning Systems (La Jolla, CA).

In addition, a mutated antibody of the present invention may be derived from a human monoclonal antibody. Such antibodies are obtained from transgenic mice that have been "engineered" to produce specific human antibodies in response to antigenic challenge. In this technique, elements of the human heavy and light chain locus are introduced into strains of mice derived from embryonic stem cell lines that contain targeted disruptions of the endogenous heavy chain and light chain loci. The transgenic mice can synthesize human antibodies specific for human antigens, and the mice can

be used to produce human antibody-secreting hybridomas. Methods for obtaining human antibodies from transgenic mice are described by Green *et al.*, *Nature Genet.* 7: 13 (1994), Lonberg *et al.*, *Nature 368*: 856 (1994), and Taylor *et al.*, *Int. Immun. 6*: 579 (1994), which are incorporated by reference.

B. Methods for Constructing a Gene Encoding a Mutated Antibody

A mutated antibody of the present invention comprises a heavy chain that contains a variable domain, a CH1 constant domain, a hinge region with multiple sulfhydryl groups and a CH3 constant domain. The lack of CH2 domain results in reduced hepatic uptake and liver toxicity, as discussed above. Moreover, the presence of CH1 and CH3 constant domains and the hinge region promotes the efficient formation of mutated antibodies *in vivo* and/or *in vitro*, and provides for the stability of the divalent structure of mutated antibody in the blood.

A suitable hinge region contains three or more sulfhydryl groups for the formation of interchain disulfide bonds. For example, the hinge region of human IgG_2 contains four interchain disulfide bonds. A preferred hinge region is obtained from human IgG_3, which contains 11 or more interchain disulfide bonds. The extended IgG_3 hinge region, described herein, contains 11 sulfhydryl groups.

DNA molecules encoding suitable variable regions, constant domains, and hinge regions can be synthesized using the polymerase chain reaction with RNA from antibody-producing hybridomas that produce such antibodies. General techniques for the synthesis of antibody components are described, for example, by Orlandi *et al.*, *Proc. Nat'l Acad. Sci. USA 86*: 3833 (1989), Larrick *et al.*, *Methods: A Companion to Methods in Enzymology 2*: 106 (1991), and by Kang *et al.*, *id.* at 111. Also see, Ward *et al.*, "Genetic Manipulation and Expression of Antibodies," in MONOCLONAL ANTIBODIES: PRINCIPLES AND APPLICATIONS, pages 137-185 (Wiley-Liss,

Inc. 1995), and Courtenay-Luck, "Genetic Manipulation of Monoclonal Antibodies," in MONOCLONAL ANTIBODIES: PRODUCTION, ENGINEERING AND CLINICAL APPLICATION, Ritter *et al.* (eds.), pages 166-179 (Cambridge University Press 1995).

Since carbohydrate groups typically are located in the CH2 domain, the mutated antibody of the present invention may lack such an attachment site. However, a glycosylation site can be introduced into the light chain variable domain, as described by Hansen *et al.*, U.S. patent No. 5,443,953 (1995). The engineered carbohydrate moiety can serve as an additional conjugation site to increase the loading capacity or as a site for conjugation of haptens that are different from those conjugated at the hinge region.

C. Methods for Expressing and Isolating the Protein Product of a Mutated Antibody DNA Sequence

To express the polypeptide encoded by the mutated antibody DNA sequence, the DNA sequence must be operably linked to regulatory sequences controlling transcriptional expression in an expression vector and then, introduced into either a prokaryotic or eukaryotic host cell. In addition to transcriptional regulatory sequences, such as promoters and enhancers, expression vectors include translational regulatory sequences and a marker gene which is suitable for selection of cells that carry the expression vector.

Suitable promoters for expression in a prokaryotic host can be repressible, constitutive, or inducible. Suitable promoters are well-known to those of skill in the art and include promoters capable of recognizing the T4, T3, Sp6 and T7 polymerases, the P_R and P_L promoters of bacteriophage lambda, the trp, recA, heat shock, and lacZ promoters of *E. coli*, the α-amylase and the σ^{28}-specific promoters of *B. subtilis*, the promoters of the bacteriophages of *Bacillus*, *Streptomyces* promoters, the *int* promoter of bacteriophage lambda, the *bla* promoter of

the β-lactamase gene of pBR322, and the CAT promoter of the chloramphenicol acetyl transferase gene. Prokaryotic promoters are reviewed by Glick, *J. Ind. Microbiol.* 1:277-282 (1987); Watson *et al.*, MOLECULAR BIOLOGY OF THE GENE, 4th Ed., Benjamin Cummins (1987); Ausubel *et al.*, *supra*, and Sambrook *et al.*, *supra*.

A preferred prokaryotic host is *E. coli*. Preferred strains of *E. coli* include Y1088, Y1089, CSH18, ER1451, and ER1647 (see, for example, Brown (Ed.), MOLECULAR BIOLOGY LABFAX, Academic Press (1991)). An alternative preferred host is *Bacillus subtilus*, including such strains as BR151, YB886, MI119, MI120, and B170 (see, for example, Hardy, "Bacillus Cloning Methods," in DNA CLONING: A PRACTICAL APPROACH, Glover (Ed.), IRL Press (1985)).

Methods for expressing antibodies in prokaryotic hosts are well-known to those of skill in the art. See, for example, Ward *et al.*, "Genetic Manipulation and Expression of Antibodies," in MONOCLONAL ANTIBODIES: PRINCIPLES AND APPLICATIONS, pages 137-185 (Wiley-Liss, Inc. 1995). Moreover, expression systems for cloning antibodies in prokaryotic cells are commercially available. For example, the IMMUNO ZAP™ Cloning and Expression System (Stratagene Cloning Systems; La Jolla, CA) provides vectors for the expression of antibody light and heavy chains in *E. coli*.

As discussed above, it may be desirable to introduce a glycosylation site into the light chain of a mutated antibody. In this case, the mutated antibody is preferably expressed in eukaryotic cells, and especially mammalian, insect, and yeast cells. Especially preferred eukaryotic hosts are mammalian cells. Mammalian cells provide post-translational modifications to the cloned polypeptide including proper folding and glycosylation. For example, such mammalian host cells include COS-7 cells (ATCC CRL 1651), non-secreting myeloma cells (SP2/0-AG14; ATCC CRL 1581), Chinese hamster ovary cells (CHO-K1; ATCC CCL 61), rat pituitary cells (GH_1; ATCC CCL

82), HeLa S3 cells (ATCC CCL 2.2), and rat hepatoma cells (H-4-II-E; ATCC CRL 1548).

For a mammalian host, the transcriptional and translational regulatory signals may be derived from viral sources, such as adenovirus, bovine papilloma virus, and simian virus. In addition, promoters from mammalian expression products, such as actin, collagen, or myosin, can be employed. Alternatively, a prokaryotic promoter (such as the bacteriophage T3 RNA polymerase promoter) can be employed, wherein the prokaryotic promoter is regulated by a eukaryotic promoter (for example, *see* Zhou *et al.*, *Mol. Cell. Biol. 10*:4529-4537 (1990); Kaufman *et al.*, *Nucl. Acids Res. 19*:4485-4490 (1991)). Transcriptional initiation regulatory signals may be selected which allow for repression or activation, so that expression of the genes can be modulated.

In general, eukaryotic regulatory regions will include a promoter region sufficient to direct the initiation of RNA synthesis. Such eukaryotic promoters include the promoter of the mouse metallothionein I gene (Hamer *et al.*, *J. Mol. Appl. Gen. 1*:273-288 (1982)); the TK promoter of Herpes virus (McKnight, *Cell 31*:355-365 (1982)); the SV40 early promoter (Benoist *et al.*, *Nature (London) 290*:304-310 (1981)); the Rous sarcoma virus promoter (Gorman *et al.*, *supra*); the cytomegalovirus promoter (Foecking *et al.*, *Gene 45*:101 (1980)); the yeast *gal4* gene promoter (Johnston, *et al.*, *Proc. Natl. Acad. Sci. (USA) 79*:6971-6975 (1982); Silver, *et al.*, *Proc. Natl. Acad. Sci. (USA) 81*:5951-5955 (1984)); and the IgG promoter (Orlandi *et al.*, *Proc. Natl. Acad. Sci. USA 86*:3833-3837 (1989)).

Strong regulatory sequences are the most preferred regulatory sequences of the present invention. Examples of such preferred regulatory sequences include the SV40 promoter-enhancer (Gorman, "High Efficiency Gene Transfer into Mammalian cells," in DNA CLONING: A PRACTICAL APPROACH, Volume II, Glover (Ed.), IRL Press pp. 143-190 (1985)), the hCMV-MIE promoter-enhancer (Bebbington *et*

al., *Bio/Technology* *10*:169-175 (1992)), and antibody heavy chain promoter (Orlandi *et al.*, *Proc. Natl. Acad. Sci. USA* *86*:3833-3837 (1989)). Also preferred are the kappa chain enhancer for the expression of the light chain and the IgH enhancer (Gillies, "Design of Expression Vectors and Mammalian Cell Systems Suitable for Engineered Antibodies," in ANTIBODY ENGINEERING: A PRACTICAL GUIDE, C. Borrebaeck (Ed.), W.H. Freeman and Company, pp. 139-157 (1992); Orlandi *et al.*, *supra*).

The mutated antibody-encoding sequence and an operably linked promoter may be introduced into eukaryotic cells as a non-replicating DNA molecule, which may be either a linear molecule or, more preferably, a closed covalent circular molecule. Since such molecules are incapable of autonomous replication, the expression of the protein may occur through the transient expression of the introduced sequence. Preferably, permanent expression occurs through the integration of the introduced sequence into the host chromosome.

Preferably, the introduced sequence will be incorporated into a plasmid or viral vector that is capable of autonomous replication in the recipient host. Several possible vector systems are available for this purpose. One class of vectors utilize DNA elements which provide autonomously replicating extra-chromosomal plasmids, derived from animal viruses such as bovine papilloma virus, polyoma virus, adenovirus, or SV40 virus. A second class of vectors relies upon the integration of the desired genomic or cDNA sequences into the host chromosome. Additional elements may also be needed for optimal synthesis of mRNA. These elements may include splice signals, as well as transcription promoters, enhancers, and termination signals. The cDNA expression vectors incorporating such elements include those described by Okayama, *Mol. Cell. Biol.* *3*:280 (1983), Sambrook *et al.*, *supra*, Ausubel *et al.*, *supra*, Bebbington *et al.*, *supra*, Orlandi *et al.*, *supra*, and Fouser *et al.*, *Bio/Technology* *10*:1121-1127 (1992);

Gillies, *supra*. Genomic DNA expression vectors which include intron sequences are described by Orlandi *et al.*, *supra*. Also, see generally, Lerner *et al.* (Eds.), NEW TECHNIQUES IN ANTIBODY GENERATION, *Methods 2(2)* (1991).

In order to obtain mammalian cells that express intact antibody, an expression vector comprising an antibody light chain can be co-transfected into mammalian cells with a mutated antibody heavy chain expression vector. See, for example, Orlandi *et al.*, *supra*. Alternatively, mammalian cells containing a mutated heavy chain expression vector can be transfected with an expression vector comprising an antibody light chain, and mammalian cells containing an expression vector comprising a light chain can be transfected with a mutated heavy chain expression vector. Moreover, mammalian cells can be transfected with a single expression vector comprising DNA fragments that encode an antibody light chain, as well as DNA fragments that encode mutated antibody heavy chain. See, for example, Gillies, *supra*; Bebbington *et al.*, *supra*. Any of these approaches will produce transfected cells that express whole antibody molecules that have the mutated heavy chain. Similar approaches can be used to produce recombinant antibodies comprising a mutated heavy chain and a light chain with an engineered glycosylation site. Standard transfection techniques are well known in the art. See, for example, Sambrook *et al.*, *supra*; Ausubel *et al.*, *supra*.

D. Methods for Isolating a Mutated Antibody from Transfected Cells

Transfected cells that carry the expression vector are selected using the appropriate drug. For example, G418 can be used to select transfected cells carrying an expression vector having the aminoglycoside phosphotransferse gene. Southern *et al.*, *J. Mol. Appl. Gen.* *1*:327-341 (1982). Alternatively, hygromycin-B can be used to select transfected cells carrying an expression vector having the hygromycin-B-

phosphotransferase gene. Palmer *et al.*, *Proc. Natl. Acad. Sci. USA* *84*:1055-1059 (1987). Alternatively, aminopterin and mycophenolic acid can be used to select transfected cells carrying an expression vector having the xanthine-guanine phosphoribosyltransferase gene. Mulligan *et al.*, *Proc. Natl. Acad. Sci. USA* *78*:2072-2076 (1981).

Transfected cells that produce a mutated antibody can be identified using a variety of methods. For example, any immunodetection assay can be used to identify such "transfectomas."

After transfectomas have been identified, the cells are cultured and antibodies are isolated from culture supernatants. Isolation techniques include affinity chromatography with Protein-A Sepharose, size-exclusion chromatography, and ion-exchange chromatography. For example, see Coligan *et al.* (eds.), CURRENT PROTOCOLS IN IMMUNOLOGY, John Wiley & Sons (1991), for detailed protocols.

4. Preparation of Immunoconjugates

Drug molecules can be attached to free thiol groups which have been generated by reduction of antibodies using standard methods for site-specific reaction at sulfur. The nucleophilic nature of the thiol group at moderate pH (pH 5 - 7) allows for protein alkylation with organic halides, epoxides, aziridines, tosylates, mesylates, maleimides, and the like, in rapid reactions that have minimal effects on other functional groups of protein. Many such alkylating agents are well-known anti-cancer drugs. Examples of alkylation drugs can be found in Holland *et al.*, CANCER MEDICINE (Lea & Fibiger 1982).

Common drugs such as doxorubicin and methotrexate have available functional groups which can be modified to carry a thiol-specific reacting group for reaction with protein. Examples of such modifications include the use of cross-linking agents such as amine/active ester or oxidation/reduction chemistries (*e.g.*, hydrazones).

These techniques are well-known to those of skill in the art. See, for example, Upeslacis *et al.*, "Modification of Antibodies by Chemical Methods," in MONOCLONAL ANTIBODIES: PRINCIPLES AND APPLICATIONS, Birch *et al.* (eds.), pages 187-230 (Wiley-Liss, Inc. 1995).

A nitrogen mustard also can be used as a hapten of an immunoconjugate. Compounds such as mechlorethamine, L-phenylalanine mustard (Melphalan), cyclophosphamide and chlorambucil can be attached to reduced mutated antibody thiol groups using an excess of drug, leaving one chloroethyl group free to react upon delivery to the target cell. Trifunctional alkylating agents such as Trenimon are particularly useful because two alkylating functions are available after the compounds are bound to the mutated antibody. Accordingly, the ability to cross-link will remain after attachment. Bizelesin, a potent bisalklyating agent, also can be utilized by reaction with a thiol-reduced mutated antibody construct in the presence of excess drug. Bizelesin and related compounds are described by Aristoff *et al.*, *DN&P 6*: 229 (1993).

Antitumor antibiotics, such as the calicheamicins and esperamicins, also are useful for the preparation of immunoconjugates. The top portion of Figure 8 illustrates the calicheamicin structure, where R' is rhamnose, R'' is an aminosugar, and R''' is a CH_3-S group that forms a trisulfide bond with sulfur. This methyl trisulfide bond plays a crucial role in the mechanism of action of the compound. The enediyene system of calicheamicin can undergo a Bergmann cyclization, forming a benzene ring with the generation of two highly reactive single electrons on the benzene ring. The triggering event for this transformation is a nucleophilic attack on the trisulfide by a thiol such as glutathione, which is present in high concentrations within cells. The free radical form of the drug can induce double-stranded DNA cleavage.

Calicheamicins and esperamicins can be bound to mutated antibodies that contain an engineered

carbohydrate group. A suitable conjugation method is described by Hinman *et al.*, *Cancer Res. 53*: 3336 (1993).

Alternatively, such compounds can be conjugated to a reduced disulfide bond of a mutated antibody. In this case, the trithiol of the drug is replaced by a mixed disulfide bond formed between the reduced disulfide bond of the antibody moiety and the drug. In Figure 8, the sulfur atom that is proximal to the R''' group would belong to a cysteine unit of the antibody moiety. The lower portion of Figure 8 illustrates how such an immunoconjugate would cleave DNA *in vivo*.

Other molecules of higher molecular weight can be coupled to mutated antibody at the hinge region to minimize the deleterious effects of conjugation at the antigen-binding region. Examples of useful high molecular weight compounds include (1) bacterial toxins (*e.g.*, *Pseudomonas* exotoxin), (2) polymers that alter the biodistribution properties of antibody conjugates (*e.g.*, polylysine, polyglutamic acid, copolymers of amino acids, (polyethylene)glycol and (polyethylene)imine, (3) substituted polymers capable of carrying increased loads of haptens such as polyamino acids (*e.g.*, polylysine, polyglutamate), starburst dendrimers, and dextrans, (4) secondary targeting vectors such as avidin/streptavidin, or single-chain polynucleotides, and (5) a second antibody, antibody fragment, or peptide that binds a different antigen.

Mutated antibodies of the present invention are particularly suitable for preparation of immunoconjugates that deliver antigenic peptides to cells for antigen presentation. See, for example, Wyss-Coray *et al.*, *Cell. Immunol. 139*: 268 (1992), which describes the use of an antibody-peptide construct to deliver antigenic peptides to T cells. In this approach, antigenic peptides are constructed with a single cysteine residue that is used to attach the peptide to the hinge region of a reduced mutated antibody via disulfide bond formation. Examples of such antigenic peptides include the tetanus toxoid

peptide p2 with an N-terminal cysteine, CQYIKANSKFIGITEL (C + tt830-844; C-ttp2; SEQ ID NO:4), and tetanus toxoid peptide p30 with a C-terminal cysteine, FNNFTVSFWLRVPKVSASHLEC (tt947-967 + C; SEQ ID NO:5). General techniques for such conjugation are well-known in the art. See, for example, Wong, CHEMISTRY OF PROTEIN CONJUGATION AND CROSS-LINKING (CRC Press 1991).

The attachment of high molecular weight polymeric materials can inhibit antibody function. Due to the larger size of polymer haptens, non-site-specific conjugation to intact antibodies or to $F(ab)_2/F(ab')_2$ fragments via lysine residues, often results in interference with antibody-antigen binding capability. Although the use of intact antibody provides the opportunity to bind a polymer to an Fc carbohydrate group, this approach also has problems. While Schiff base forming reactions work well for hydrazide derivatives, the reactions are less effective for amino derivatives because 100 - 1000 fold molar excesses of amino derivatives are often needed to drive the reaction to completion. See, for example, Rodwell *et al.*, *Proc. Nat'l Acad. Sci. USA 83*: 2632 (1985). Consequently, a sufficient amount of hapten may not be available for coupling to an oxidized carbohydrate group for costly amino group-bearing polymers such as streptavidin, *Pseudomonas* exotoxin and ribonuclease.

The hinge region thiol moieties of the mutated antibodies described herein provide the site-specificity required for polymer conjugation. Unexpectedly, it was found that intrinsic thiol groups (*i.e.*, those derived from reduced Mab disulfide bonds) react differently than "appended" thiol groups that have been generated by substitution of thiolation reagents onto Mab lysine residues. This point is illustrated by Figure 2 which shows two, superimposed preparative size-exclusion high-pressure liquid chromatography (HPLC) traces. In this study, hIMMU-14 antibody, a high-affinity Mab raised against (CEA), was used as a model.

In Figure 2, the background trace shows hIMMU-14-IgG-streptavidin prepared by reaction of thiolated antibody and maleimido-streptavidin wherein the sulfhydryl groups are appended to the Mab by 2-iminothiolane (Traut's reagent). The smaller foreground trace shows hIMMU-14-IgG-streptavidin prepared by reaction of reduced antibody and maleimido-streptavidin wherein the sulfhydryl groups are derived by reduction of Mab disulfide bonds. Lower weight materials are on the left-hand side, with the peak at the farthest left being the unreacted hIMMU-14 Mab. The peak to the immediate right of the Mab peak is the desired conjugate, hIMMU-14-streptavidin.

The background trace shows a clear discrimination between the Mab and the hIMMU-14-streptavidin peak, whereas the foreground trace shows the hIMMU-14-streptavidin peak appearing as a shoulder on the Mab peak. This observation indicates that the hIMMU-14-streptavidin prepared via reduction of intrinsic disulfide bonds forms a species of lower apparent molecular weight than the same reagent prepared using a thiolation reagent, suggesting that, in the former case, the two proteins must come closer together so that reaction with the buried thiol groups can occur. The result is a tighter fit protein-protein conjugate, as opposed to two distinct proteins held apart by a relatively long-chain organic linker. The tighter-fit protein-protein conjugates are expected to be less recognizable by the reticuloendothelial cell system, behaving *in vivo* more as single proteinaceous entities. Consequently, these conjugates are expected to have longer circulation times and superior biodistribution properties.

Metals useful in diagnosis and therapy can be attached to a reduced hinge region of a mutated antibody. Chelators for radiometals or magnetic resonance enhancers are well-known in the art. Typical are derivatives of ethylenediaminetetraacetic acid (EDTA) and diethylenetriaminepentaacetic acid (DTPA). These

chelators typically have groups on the side chain by which the chelator can be attached to a carrier. Such groups include, *e.g.*, benzylisothiocyanate, by which the DTPA or EDTA can be coupled to the amine group of a carrier. Alternatively, carboxyl groups or amine groups on a chelator can be coupled to a carrier by activation or prior derivatization and then coupling, all by well-known means.

Labels such as enzymes, fluorescent compounds, electron transfer agents, and the like can be linked to a carrier by conventional methods well known to the art. These labeled carriers and the immunoconjugates prepared from them can be used for immunochemical detection, as described below.

Boron addends, such as carboranes, can be attached to antibody components by conventional methods. For example, carboranes can be prepared with carboxyl functions on pendant side chains, as is well known in the art. Attachment of such carboranes to a carrier, *e.g.*, aminodextran, can be achieved by activation of the carboxyl groups of the carboranes and condensation with amines on the carrier to produce an intermediate conjugate. Such intermediate conjugates are then attached to antibody components to produce therapeutically useful immunoconjugates, as described below.

Alternatively, carboranes can be attached to mutated antibodies via pendent thiol groups. For example, borocaptate salts can be coupled via mixed disulfide bond formation with reduced mutated antibody, or by conversion to a maleimide derivative by reaction with excess bis(maleimide) cross-linkers, such as bis(maleimide)hexane. The second maleimido group is then used for reaction with reduced mutated antibody. The first method produces a cleavably linked carborane, while the second approach provides a carborane that is not cleavable from the antibody moiety.

As discussed above, it may be desirable to introduc a carbohydrate moiety into the light chain variable region of a mutated antibody. The carbohydrate group can be used to increase the loading of the same hapten that is bound to a thiol group, or the carbohydrate moiety can be used to bind a different hapten. For example, the carbohydrate moiety can be used to attach polyethyleneglycol in order to extend the half-life of a mutated antibody in blood, lymph, or other extracellular fluids.

Methods for conjugating haptens to antibody components via an antibody carbohydrate moiety are well-known to those of skill in the art. See, for example, Shih *et al.*, *Int. J. Cancer* *41*:832-839 (1988); Shih *et al.*, *Int. J. Cancer* *46*:1101-1106 (1990); Shih *et al.*, U.S. patent No. 5,057,313, and Hansen *et al*, U.S. patent No. 5,443,953 (1995). The general method involves reacting an antibody component having an oxidized carbohydrate portion with a carrier polymer that has at least one free amine function and that is loaded with a plurality of drug, toxin, chelator, boron addends, or other diagnostic or therapeutic agent. This reaction results in an initial Schiff base (imine) linkage, which can be stabilized by reduction to a secondary amine to form the final conjugate.

5. Use of Immunoconjugates for Diagnosis

A. *In Vitro* Diagnosis

The present invention contemplates the use of immunoconjugates to screen biological samples *in vitro* for the expression of tumor associated antigens or for the presence of antigens associated with infectious agents. For example, the immunoconjugates of the present invention can be used to detect the presence of CEA in tissue sections prepared from a biopsy specimen. General immunochemistry techniques are well-known to those of ordinary skill. See, for example, Ponder, "Cell Marking Techniques and Their Application," in MAMMALIAN DEVELOPMENT: A PRACTICAL APPROACH, Monk (ed.), pages 115-

38 (IRL Press 1987), Volm *et al.*, *Eur. J. Cancer Clin. Oncol.* *25*: 743 (1989), Coligan at pages 5.8.1-5.8.8, and Ausubel *et al.* (eds.), CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, pages 14.6.1 to 14.6.13 (Wiley Interscience 1990). Also, see generally, Manson (ed.), METHODS IN MOLECULAR BIOLOGY, VOL.10: IMMUNOCHEMICAL PROTOCOLS (The Humana Press, Inc. 1992).

Moreover, immunochemical detection techniques can be used to optimize mutated antibodies for subsequent *in vivo* diagnosis and therapy. For example, an antibody moiety that binds the *c-erb* B2 proto-oncogene product may be more suitable for a particular breast cancer than an antibody moiety that binds carcinoembryonic antigen.

Immunochemical detection can be performed by contacting a biological sample with a mutated antibody and then contacting the biological sample with a detectably labeled molecule which binds to the mutated antibody. For example, the detectably labeled molecule can comprise an antibody moiety that binds the mutated antibody. Alternatively, the mutated antibody can be conjugated with avidin/streptavidin (or biotin) and the detectably labeled molecule can comprise biotin (or avidin/streptavidin). Numerous variations of this basic technique are well-known to those of skill in the art.

Alternatively, a mutated antibody can be conjugated with a diagnostic agent to form an immunoconjugate. Antibodies can be detectably labeled with any appropriate marker moiety, for example, a radioisotope, a fluorescent label, a chemiluminescent label, an enzyme label, a bioluminescent label or colloidal gold. Methods of making and detecting such detectably-labeled immunoconjugates are well-known to those of ordinary skill in the art, and are described in more detail below.

The marker moiety can be a radioisotope that is detected by autoradiography. Isotopes that are particularly useful for the purpose of the present invention are ^{3}H, ^{125}I, ^{131}I, ^{35}S and ^{14}C.

Immunoconjugates also can be labeled with a fluorescent compound. The presence of a fluorescently-labeled antibody component is determined by exposing the immunoconjugate to light of the proper wavelength and detecting the resultant fluorescence. Fluorescent labeling compounds include fluorescein isothiocyanate, rhodamine, phycoerytherin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

Alternatively, immunoconjugates can be detectably labeled by coupling to a chemiluminescent compound. The presence of the chemiluminescent-tagged immunoconjugate is determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of chemiluminescent labeling compounds include luminol, isoluminol, an aromatic acridinium ester, an imidazole, an acridinium salt and an oxalate ester.

Similarly, a bioluminescent compound can be used to label immunoconjugates of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Bioluminescent compounds that are useful for labeling include luciferin, luciferase and aequorin.

Alternatively, immunoconjugates can be detectably labeled by linking a mutated antibody to an enzyme. When the mutated antibody-enzyme conjugate is incubated in the presence of the appropriate substrate, the enzyme moiety reacts with the substrate to produce a chemical moiety which can be detected, for example, by spectrophotometric, fluorometric or visual means. Examples of enzymes that can be used to detectably label immunoconjugates include β-galactosidase, glucose oxidase, peroxidase and alkaline phosphatase.

Those of skill in the art will know of other suitable labels which can be employed in accordance with the present invention. The binding of marker moieties to

antibody components can be accomplished using standard techniques known to the art. Typical methodology in this regard is described by Kennedy *et al.*, *Clin. Chim. Acta* 70: 1 (1976), Schurs *et al.*, *Clin. Chim. Acta* 81: 1 (1977), Shih *et al.*, *Int'l J. Cancer* 46: 1101 (1990), Stein *et al.*, *Cancer Res. 50*: 1330 (1990), *supra*, and Stein *et al.*, *Int. J. Cancer 55*: 938 (1993). Also, see generally, Coligan.

In addition, the convenience and versatility of immunochemical detection can be enhanced by using mutated antibodies that have been conjugated with avidin, streptavidin, and biotin. See, for example, Wilchek *et al.* (eds.), *Avidin-Biotin Technology*, METHODS IN ENZYMOLOGY, VOL. 184 (Academic Press 1990), and Bayer *et al.*, "Immunochemical Applications of Avidin-Biotin Technology," in METHODS IN MOLECULAR BIOLOGY, VOL. 10, Manson (ed.), pages 149-162 (The Human Press, Inc. 1992).

Thus, the above-described immunochemical detection methods can be used to assist in the diagnosis or staging of a pathological condition. These techniques also can be used to identify the most suitable composition of mutated antibody immunoconjugate for subsequent *in vivo* diagnosis and therapy.

B. *In Vivo* Diagnosis

The present invention also contemplates the use of immunoconjugates for *in vivo* diagnosis. As an illustration, immunoconjugates can be used to diagnose cardiovascular disease. For example, immunoconjugates comprising anti-mysosin fragments can be used for imaging myocardial necrosis associated with acute myocardial infarction. Immunoconjugates comprising an antibody component that binds platelets and fibrin can be used for imaging deep-vein thrombosis. Moreover, immunoconjugates that bind to activated platelets can be used for imaging atherosclerotic plaque. Furthermore, immunoconjugates of the present invention can be used to localize particular tumors and infectious agents.

The method of diagnostic imaging with radiolabeled Mabs is well-known. In the technique of immunoscintigraphy, for example, antibodies are labeled with a gamma-emitting radioisotope and introduced into a patient. A gamma camera is used to detect the location and distribution of gamma-emitting radioisotopes. See, for example, Srivastava (ed.), RADIOLABELED MONOCLONAL ANTIBODIES FOR IMAGING AND THERAPY (Plenum Press 1988), Chase, "Medical Applications of Radioisotopes," in REMINGTON'S PHARMACEUTICAL SCIENCES, 18th Edition, Gennaro *et al.* (eds.), pp. 624-652 (Mack Publishing Co., 1990), Brown, "Clinical Use of Monoclonal Antibodies," *in* BIOTECHNOLOGY AND PHARMACY 227-49, Pezzuto *et al.* (eds.) (Chapman & Hall 1993), Goldenberg, *CA - A Cancer Journal for Clinicians* 44: 43 (1994), and Bamias *et al.*, "Monoclonal Antibodies in Oncology: *In Vivo* Targeting for Immunoscintigraphy and Therapy of Human Malignancies," in MONOCLONAL ANTIBODIES: PRODUCTION, ENGINEERING AND CLINICAL APPLICATION, Ritter *et al.* (eds.), pages 222-246 (Cambridge University Press 1995).

For diagnostic imaging, radioisotopes may be bound to a mutated antibody either directly, or indirectly by using an intermediary functional group. Bifunctional chelates can be attached to hinge region thiols using the alkylation reactions described above. Examples of such agents include bromoacetyl- or maleimide derivatives such as 2-(p-bromoacetylamido)benzyl-diethylenetriaminepentaacetic acid (DTPA), and 2-(p-bromoacetylamido)benzyl-1,4,7,10-tetraazadodecane-N,N', N'', N'''-tetraacetic acid (DOTA).

Certain metals can be bound directly to the free thiol groups in a stable manner to give useful metalloproteins, including chemically reduced technetium-99m, technetium-94m and rhenium isotopes, as well as "soft" metals such as silver-111 and the copper isotopes. Site-specific attachment of these metals to mutated antibody via bifunctional chelating agents or directly onto reduced hinge region disulfide bonds allows for

labeling of the mutated antibody with these elements in procedures which are amenable to an easy-to-use kit formulation. For example, see Shih *et al.*, *supra*, and U.S. patent No. 5,057,313. Also, see Griffiths, U.S. patent No. 5,128,119 (1992).

The radiation dose delivered to the patient is maintained at as low a level as possible through the choice of isotope for the best combination of minimum half-life, minimum retention in the body, and minimum quantity of isotope which will permit detection and accurate measurement. Examples of radioisotopes that can be bound to antibodies and are appropriate for diagnostic imaging include γ-emitters and positron-emitters such as ^{99m}Tc, ^{94m}Tc, ^{67}Ga, ^{64}Cu, ^{111}In, ^{123}I, ^{124}I, ^{125}I, ^{131}I, ^{51}Cr, ^{89}Zr, ^{18}F and ^{68}Ga. Other suitable radioisotopes are known to those of skill in the art.

Preferred γ-emitters have a gamma radiation emission peak in the range of 50-500 Kev, primarily because the state of the art for radiation detectors currently favors such labels. Examples of such γ-emitters include ^{99m}Tc, ^{67}Ga, ^{123}I, ^{125}I and ^{131}I.

Significantly, the mutated antibodies of the present invention overcome practical problems associated with the use of rhenium-186 and silver-111. Rhenium-186 is a carrier-added nuclide and the ability to load multiple radiometal units at an antigen-distant site is a significant advantage over other methodologies used for rhenium labeling of Mabs. Multiple loading of silver-111 (and numerous other similarly produced nuclides) onto a mutated antibody also confers a significant additional practical advantage to this type of conjugate because the major factor limiting use of this radiometal is the cost of production from isotopically-enriched and expensive palladium-110. Multiple loading of radiometal allows for the use of silver-111 and similar nuclides at lower specific activity. Therefore, silver-111 produced by irradiation of less expensive natural palladium becomes a more practical proposition.

Antibodies also can be labeled with paramagnetic ions for purposes of *in vivo* diagnosis. Elements that are particularly useful for magnetic resonance imaging include Gd, Mn, Dy and Fe ions.

A high background level of non-targeted antibody provides a major impediment to *in vivo* diagnosis methodology. However, the ratio of target to nontarget radiolabeled antibody can be enhanced through the use of a nonlabeled second antibody which scavenges and promotes the clearance of the nontargeted circulating radiolabeled antibody. The second antibody may be whole IgG or IgM, or a fragment of IgG or IgM, so long as it is capable of binding the radiolabeled antibody to form a complex which is cleared from the circulation and nontarget spaces more rapidly than the radiolabeled antibody alone. In the present context, suitable second antibodies may bind with either the Fc portion (*i.e.*, CH3 domain) or variable region of a radiolabeled immunoconjugate. See, for example, Goldenberg, U.S. patent No. 4,624,846, Goldenberg, international publication No. WO 92/19273, and Sharkey *et al.*, *Int. J. Cancer 51*: 266 (1992), which are incorporated by reference.

For example, the location of CEA-bearing tumor cells can be determined by parenterally injecting a mammal with an immunoconjugate comprising a variable domain that binds with CEA and a diagnostic agent. Subsequently, the mammal is injected with an antibody or antibody fragment that binds with the immunoconjugate in an amount that is sufficient to decrease the level of circulating immunoconjugate by about 10-85% within 2 to 72 hours. The mammal is then scanned with a detector to locate the site or sites of uptake of the immunoconjugate. See Goldenberg, U.S. patent No. 4,624,846.

In an alternate approach, detection methods are improved by taking advantage of the binding between avidin/streptavidin and biotin. Avidin, found in egg whites, has a very high binding affinity for biotin, which is a B-complex vitamin. Streptavidin, isolated

from *Streptomyces avidinii*, is similar to avidin, but has lower non-specific tissue binding and therefore, streptavidin often is used in place of avidin. A basic diagnostic method comprises administering a mutated antibody conjugated with avidin/streptavidin (or biotin), injecting a clearing composition comprising biotin (or avidin/streptavidin), and administering a conjugate of a diagnostic agent and biotin (or avidin/streptavidin). Preferably, the biotin (or avidin/streptavidin) component of the clearing composition is coupled with a carbohydrate moiety (such as dextran) or a polyol group (*e.g.*, polyethylene glycol) to decrease immunogenicity and permit repeated applications.

A modification of the basic method is performed by parenterally injecting a mammal with a mutated antibody that has been conjugated with avidin/streptavidin (or biotin), injecting a clearing composition comprising biotin (or avidin/streptavidin), and parenterally injecting an immunoconjugate according to the present invention, which further comprises avidin/streptavidin (or biotin). See Goldenberg, international publication No. WO 94/04702, which is incorporated by reference.

In a further variation of this method, improved detection can be achieved by conjugating multiple avidin/streptavidin or biotin moieties to a polymer which, in turn, is conjugated to an antibody component. Adapted to the present invention, immunoconjugates can be produced which contain multiple avidin/streptavidin or biotin moieties. Techniques for constructing and using multiavidin/multistreptavidin and/or multibiotin polymer conjugates to obtain amplification of targeting are disclosed by Griffiths, international application No. PCT/US94/04295, which is incorporated by reference.

In another variation, improved detection is achieved by injecting a targeting mutated antibody conjugated to biotin (or avidin/streptavidin), injecting at least one dose of an avidin/streptavidin (or biotin) clearing agent, and injecting a diagnostic composition comprising

a conjugate of biotin (or avidin/streptavidin) and a naturally occurring metal atom chelating protein which is chelated with a metal detection agent. Suitable targeting proteins according to the present invention would be ferritin, metallothioneins, ferredoxins, and the like. This approach is disclosed by Goldenberg *et al.*, international application No. PCT/US94/05149, which is incorporated by reference.

Those of skill in the art are aware that certain antibodies internalize into cells very rapidly, while others internalize into cells very slowly, or not at all. An example of the latter type is the hIMMU-14 antibody, a high-affinity Mab raised against carcinoembryonic antigen. Mutated antibody constructs of the hIMMU-14 Mab are most usefully coupled to a protein such as streptavidin, and then used for localizing biotin-isotope/drug conjugates to the target, since such as two-step delivery system requires an initial targeting agent which remains on the outside of the targeted cell.

Immunoconjugates comprising a radiolabel also can be used to detect tumor cells or infectious agents in the course of intraoperative and endoscopic examination using a small radiation detection probe. See Goldenberg U.S. patent No. 4,932,412, which is incorporated by reference. As an illustration of the basic approach, a surgical or endoscopy subject is injected parenterally with an immunoconjugate comprising a variable domain that binds with an antigen that is associated with a tumor or infectious agent and a radioisotope. Subsequently, the surgically exposed or endoscopically accessed interior of the body cavity of the subject is scanned at close range with a radiation detection probe to detect the sites of accretion of the immunoconjugate.

In a variation of this method, a photoactive agent or dye, such as dihematoporphyrin ether (Photofrin II), is injected systemically and sites of accretion of the agent or dye are detected by laser-induced fluorescence and endoscopic imaging. See Goldenberg, international

application No. PCT/US93/04098, which is incorporated by reference. The prior art discloses imaging techniques using certain dyes that are accreted by lesions, such as tumors, and which are in turn activated by a specific frequency of light. These methods are described, for example, in Dougherty *et al.*, *Cancer Res. 38*: 2628 (1978); Dougherty, *Photochem. Photobiol. 45*: 879 (1987); Doiron *et al.* (eds.), PORPHYRIN LOCALIZATION AND TREATMENT OF TUMORS (Alan Liss, 1984); and van den Bergh, *Chem. Britain 22*: 430 (1986), which are incorporated herein in their entirety by reference.

In a basic technique, a subject is injected parenterally with an immunoconjugate comprising a variable domain that binds with an antigen that is associated with a tumor or infectious agent and a photoactive agent or dye. Sites of accretion are detected using a light source provided by an endoscope or during a surgical procedure.

The detection of immunoconjugates during intraoperative or endoscopic examination can be enhanced through the use of second antibody or avidin/streptavidin/biotin clearing agents, as discussed above.

In these endoscopic techniques the detection means can be inserted into a body cavity through an orifice, such as, the mouth, nose, ear, anus, vagina or incision. As used herein, the term "endoscope" is used generically to refer to any scope introduced into a body cavity, *e.g.*, an anally introduced endoscope, an orally introduced bronchoscope, a urethrally introduced cystoscope, an abdominally introduced laparoscope or the like. Certain of these may benefit greatly from further progress in miniaturization of components and their utility to practice the method of the present invention will be enhanced as a function of the development of suitably microminiaturized components for this type of instrumentation. Highly miniaturized probes which could be introduced intravascularly, *e.g.*, via catheters or the

like, are also suitable for use in the embodiments of the invention for localizing tumor cells or infectious agents.

6. Use of Immunoconjugates for Therapy

The present invention also contemplates the use of immunoconjugates for immunotherapy. An objective of immunotherapy is to deliver cytotoxic doses of radioactivity, toxin, or drug to target cells, while minimizing exposure to non-target tissues. The immunoconjugates of the present invention are expected to combine the high antigen-binding capacity and high hapten-binding capacity of intact antibodies with the rapid clearance and high tissue penetrability of antibody fragments.

For example, a therapeutic immunoconjugate may comprise an α-emitting radioisotope, a β-emitting radioisotope, a γ-emitting radioisotope, an Auger electron emitter, a neutron capturing agent that emits α-particles or a radioisotope that decays by electron capture. Suitable radioisotopes include ^{198}Au, ^{32}P, ^{125}I, ^{131}I, ^{90}Y, ^{186}Re, ^{188}Re, ^{67}Cu, ^{211}At, and the like.

As discussed above, a radioisotope can be attached to an antibody directly or indirectly, via a chelating agent. For example, ^{67}Cu, considered one of the more promising radioisotopes for radioimmunotherapy due to its 61.5 hour half-life and abundant supply of beta particles and gamma rays, can be conjugated to an antibody using the chelating agent, p-bromoacetamido-benzyl-tetraethylaminetetraacetic acid (TETA). Chase, *supra*. Alternatively, ^{90}Y, which emits an energetic beta particle, can be coupled to an antibody using diethylenetriaminepentaacetic acid (DTPA).

Alternatively, boron addends such as carboranes can be attached to mutated antibodies. Carboranes can be prepared with carboxyl functions on pendant side chains, as is well-known in the art. Attachment of carboranes to a carrier, such as aminodextran, can be achieved by activation of the carboxyl groups of the carboranes and

condensation with amines on the carrier. The intermediate conjugate is then conjugated to the antibody. After administration of the immunoconjugate, a boron addend is activated by thermal neutron irradiation and converted to radioactive atoms which decay by α-emission to produce highly toxic, short-range effects.

In addition, therapeutically useful immunoconjugates can be prepared in which a mutated antibody is conjugated to a toxin or a chemotherapeutic drug. Illustrative of toxins which are suitably employed in the preparation of such conjugates are ricin, abrin, human ribonuclease, pokeweed antiviral protein, gelonin, diphtherin toxin, and *Pseudomonas* endotoxin. See, for example, Pastan *et al.*, *Cell* 47: 641 (1986), and Goldenberg, *CA - A Cancer Journal for Clinicians* 44: 43 (1994). Other suitable toxins are known to those of skill in the art.

Useful cancer chemotherapeutic drugs for the preparation of immunoconjugates include nitrogen mustards, alkyl sulfonates, nitrosoureas, triazenes, folic acid analogs, pyrimidine analogs, purine analogs, antibiotics, epipodophyllotoxins, platinum coordination complexes, hormones, and the like. Chemotherapeutic drugs that are useful for treatment of infectious agents include antiviral drugs (such as AZT, 2',3'-dideoxyinosine and 2',3'-dideoxycytidine), antimalarial drugs (such as chloroquine and its congeners, diaminopyrimidines, mefloquine), antibacterial agents, antifungal agents, antiprotozoal agents, and the like. Suitable chemotherapeutic agents are described in REMINGTON'S PHARMACEUTICAL SCIENCES, 18th Ed. (Mack Publishing Co. 1990), and in GOODMAN AND GILMAN'S THE PHARMACOLOGICAL BASIS OF THERAPEUTICS, 7th Ed. (MacMillan Publishing Co. 1985), which are incorporated by reference. Other suitable chemotherapeutic agents, such as experimental drugs, are known to those of skill in the art.

Immunoconjugates of the present invention also can be used for prodrug therapy. In this approach, a mutated antibody-enzyme conjugate is used to localize the enzyme to target tissue. Subsequent administration of prodrug results in the site-specific activation of the prodrug at target cells.

In addition, therapeutically useful immunoconjugates can be obtained by conjugating photoactive agents or dyes to a mutated antibody. Fluorescent and other chromogens, or dyes, such as porphyrins sensitive to visible light, have been used to detect and to treat lesions by directing the suitable light to the lesion (cited above). In therapy, this has been termed photoradiation, phototherapy, or photodynamic therapy (Jori *et al.* (eds.), PHOTODYNAMIC THERAPY OF TUMORS AND OTHER DISEASES (Libreria Progetto 1985); van den Bergh, *Chem. Britain 22*: 430 (1986)). Moreover, antibodies have been coupled with photoactivated dyes for achieving phototherapy (Mew *et al., J. Immunol. 130*: 1473 (1983); *idem., Cancer Res. 45*: 4380 (1985); Oseroff *et al., Proc. Natl. Acad. Sci. USA 83*: 8744 (1986); *idem., Photochem. Photobiol. 46*: 83 (1987); Hasan *et al., Prog. Clin. Biol. Res. 288*: 471 (1989); Tatsuta *et al., Lasers Surg. Med. 9*: 422 (1989); Pelegrin *et al., Cancer 67*: 2529 (1991) - all incorporated in their entirety herein by reference). Thus, the present invention contemplates the therapeutic use of immunoconjugates comprising photoactive agents or dyes. The general methodology is described above in relation to the use of such immunoconjugates for diagnosis.

The present invention also contemplates two-, three- or four-step targeting strategies to enhance antibody therapy. General techniques include the use of mutated antibodies conjugated with avidin, streptavidin or biotin, and the use of second antibodies that bind with the primary immunoconjugate, as discussed above. See, for example, Goodwin *et al., Eur. J. Nucl. Med. 9*:209 (1984), Goldenberg *et al., J. Nucl. Med. 28*:1604 (1987),

Hnatowich *et al.*, *J. Nucl. Med.* *28*: 1294 (1987), Paganelli *et al.*, *Cancer Res.* *51*: 5960 (1991), Goldenberg, international publication No. WO 92/19273, Sharkey *et al.*, *Int. J. Cancer* *51*: 266 (1992), and Goldenberg, international application No. WO 94/04702, which are incorporated by reference. Also, see Griffiths, international application No. PCT/US94/04295, which describes a method using multiavidin and/or multibiotin polymer conjugates, and Goldenberg *et al.*, international application No. PCT/US94/05149, which discloses improved methods for therapy with chelatable radiometals.

For example, a mammal having a disease caused by an infectious agent may be treated by parenterally injecting the mammal with an immunoconjugate comprising a variable domain that binds with an antigen that is associated with the infectious agent and a therapeutic agent. Subsequently, the mammal is injected with an antibody or antibody fragment that binds with the immunoconjugate in an amount that is sufficient to decrease the level of circulating immunoconjugate by about 10-85% within 2 to 72 hours.

An alternative approach to enhancing the therapeutic index comprises administering a mutated antibody conjugated with avidin/streptavidin (or biotin), injecting a clearing composition comprising biotin (or avidin/streptavidin), and administering a conjugate of a therapeutic agent and avidin/streptavidin (or biotin), as discussed above.

Those of skill in the art are aware that the therapeutic index can also be enhanced by the choice of the antibody component. For example, LL2 is a murine Mab that has been shown to be effective for the diagnosis and treatment of non-Hodgkins B-cell lymphoma. Goldenberg *et al.*, *J. Clin. Oncol.* *9*: 548 (1991); Murthy *et al.*, *Eur. J. Nucl. Med.* *19*: 394 (1992). Once bound to antigen, this Mab is internalized very rapidly, making a mutated antibody construct of this Mab very useful with protein

agents such as ribonuclease and deoxyribonuclease, which can cleave RNA and DNA, respectively, in the target cell. Similarly, in the case of the drug conjugates of a mutated form of LL2, an internalizing construct would be expected to have superior therapeutic properties.

Immunoconjugates may be administered alone, or in conjugation with the conventional chemotherapeutic agents described above. Modes of chemotherapeutic administration and suitable dosages are well known to those of skill in the art. See, for example, REMINGTON'S PHARMACEUTICAL SCIENCES, 18th Ed. (Mack Publishing Co. 1990), and GOODMAN AND GILMAN'S THE PHARMACOLOGICAL BASIS OF THERAPEUTICS, 7th Ed. (MacMillan Publishing Co. 1985).

In general, the dosage of administered immunoconjugates will vary depending upon such factors as the patient's age, weight, height, sex, general medical condition and previous medical history. Typically, it is desirable to provide the recipient with a dosage of immunoconjugate which is in the range of from about 1 pg/kg to 10 mg/kg (amount of agent/body weight of patient), although a lower or higher dosage also may be administered as circumstances dictate.

Administration of immunoconjugates to a patient can be intravenous, intraarterial, intraperitoneal, intramuscular, subcutaneous, intrapleural, intrathecal, by perfusion through a regional catheter, or by direct intralesional injection. When administering immunoconjugates by injection, the administration may be by continuous infusion or by single or multiple boluses.

Immunoconjugates having a boron addend-loaded carrier for thermal neutron activation therapy will normally be effected in similar ways. However, it will be advantageous to wait until non-targeted immunoconjugate clears before neutron irradiation is performed. Clearance can be accelerated using an antibody that binds to the immunoconjugate. See U.S. patent No. 4,624,846 for a description of this general principle.

The immunoconjugates of the present invention can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby immunoconjugates are combined in a mixture with a pharmaceutically acceptable carrier. A composition is said to be a "pharmaceutically acceptable carrier" if its administration can be tolerated by a recipient patient. Sterile phosphate-buffered saline is one example of a pharmaceutically acceptable carrier. Other suitable carriers are well-known to those in the art. See, for example, REMINGTON'S PHARMACEUTICAL SCIENCES, 18th Ed. (1990).

For purposes of therapy, an immunoconjugate and a pharmaceutically acceptable carrier are administered to a patient in a therapeutically effective amount. A combination of an immunoconjugate and a pharmaceutically acceptable carrier is said to be administered in a "therapeutically effective amount" if the amount administered is physiologically significant. An agent is physiologically significant if its presence results in a detectable change in the physiology of a recipient patient. In the present context, an agent is physiologically significant if its presence results in the inhibition of the growth of target cells.

Additional pharmaceutical methods may be employed to control the duration of action of an immunoconjugate in a therapeutic application. Control release preparations can be prepared through the use of polymers to complex or adsorb the immunoconjugate. For example, biocompatible polymers include matrices of poly(ethylene-co-vinyl acetate) and matrices of a polyanhydride copolymer of a stearic acid dimer and sebacic acid. Sherwood *et al.*, *Bio/Technology* 10: 1446 (1992). The rate of release of an immunoconjugate from such a matrix depends upon the molecular weight of the immunoconjugate, the amount of immunoconjugate within the matrix, and the size of dispersed particles. Saltzman *et al.*, *Biophys. J.* 55: 163 (1989); Sherwood *et al.*, *supra*. Other solid dosage

forms are described in REMINGTON'S PHARMACEUTICAL SCIENCES, 18th ed. (1990).

The present invention, thus generally described, will be understood more readily by reference to the following examples, which are provided by way of illustration and are not intended to be limiting of the present invention.

Example 1

Preparation of Mutated Antibodies Containing Multiple Disulfide Bonds

The DNA sequence encoding the human IgG_1 hinge region and the CH2 domain is replaced with the human IgG_3 hinge region as follows. A 1.6 kilobase *Bam*HI/*Eag*I fragment containing the genomic sequence encoding the human IgG_1 constant region is excised from the heavy chain expression vector, LL2pG1g, and subcloned into the corresponding sites of a derivative of pBLUESCRIPT SK (Stratagene; La Jolla, CA) that lacks the *Pst*I cloning site. Leung *et al.*, *Hybridoma 13*: 469 (1994). See Figure 3.

A staging vector, designated as CHpBSK, is shown in Figure 4. Within the 1.6 kilobase immunoglobulin fragment in CHpBSK, there are unique *Pst*I and *Sfi*I sites that flank the genomic DNA sequences encoding the IgG_1 hinge region and the CH2 domain. The *Pst*I site is located in the intron sequence immediately adjacent and upstream of the exon sequence encoding the IgG_1 hinge region, while the *Sfi*I site is located in the intron sequence about 35 base pairs downstream of the exon sequence encoding the CH2 domain. The fragment encoding the human IgG_1 hinge region and CH2 domain is excised from CHpBSK by digestion with *Pst*I and *Sfi*I. A DNA fragment containing the genomic sequence for human IgG_3 hinge region is subsequently inserted into this site, replacing the IgG_1 hinge region and the CH2 domain, as follows.

The human IgG_3 hinge region amino acid sequence is: ELKTPLGDTT HTCPRCPEPK SCDTPPPCPR CPEPKSCDTP PPCPRCPEPK SCDTPPPCPR CP [SEQ ID NO:1]. Kabat *et al.*, SEQUENCES OF PROTEINS OF IMMUNOLOGICAL INTEREST, VOLUME II (1991). A DNA fragment encoding the human IgG_3 hinge region is obtained by polymerase chain reaction (PCR) as follows. The construct, pdHL2-Immγ3Fab (Immunomedics, Inc.; Morris Plains, NJ), containing the constant region genomic sequence of human IgG_3 is used as the template for the PCR-amplification by the primer pairs, h3BACK(+) and h3FOR(-). The h3BACK(+) primer is a 39-mer with the nucleotide sequence of 5'-ctt ctc tct gca gAG CTC AAA ACC CCA CTT GGT GAC ACA-3' [SEQ ID NO:2], where lower case letters represent the intron sequence, uppercase letters represent the sequence encoding the first nine amino acids of the human IgG_3 hinge region, and the underlined portion indicates the *PstI* site at the intron/exon junction. The antisense primer h3FOR(-) is a 63-mer with the nucleotide sequence of 5'-ggt ggg ccg agc cgg cct ggc cct cgc acc cca cgg gtc cca cCT TTG GCT TTG GAG ATG GTT-3' [SEQ ID NO: 3], where lower case letters represent the intron sequence, uppercase letters represent the sequence encoding the last six amino acids of the human IgG_3 hinge region, and the underlined portion indicates the *SfiI* site.

The PCR reactions are performed in a volume of 100 μl by mixing 3 μg of pdHL2-Immγ3Fab with 5 μl of 5 μM of the flanking primer pairs in the presence of 10 μl of 10x PCR buffer (500 mM KCl, 100 mM Tris-HCl (pH 8.3), 15 mM $MgCl_2$) and 5 units of AmpliTaq DNA polymerase (Perkin Elmer Cetus; Norwalk, CT). The reaction mixture is subjected to 30 cycles of PCR reaction consisting of denaturation at 94°C for one minute, annealing at 55°C for 1.5 minutes, and polymerization at 72°C for 1.5 minutes. Double-stranded PCR-amplified products (about 650 base pairs in length) are gel-purified, digested with *PstI* and *SfiI*, and subcloned into the complementary sites of CHpBSK. The resultant construct, designated as

ΔC2h3pBSK (Figure 4), contains sequences encoding a human constant region with the structure of [CH1(IgG_1)]-[IgG_3 hinge]-[CH3 (IgG_1)]. Sanger's dideoxy sequencing reactions are carried out to confirm the sequence.

The human Δ$C2h3IgG_1$ constant region fragment is excised from ΔC2h3pBSK by digestion with *Bam*HI and *Eag*I and subcloned into the corresponding sites of the vector hMN14pG1g, replacing the human IgG_1 constant region. Leung *et al.*, *Hybridoma 13*: 469 (1994); Leung *et al.*, *J. Immunol. 154*: 5919 (1995). The resultant expression vector, hMN14-ΔC2h3pG1g (Figure 5), when co-transfected with the light-chain expression vector, hMN14pKh, into SP2/0 cells will express a divalent hMN14 antibody having a long hinge region and lacking a CH2 domain. Co-transfection conditions are described by Leung *et al.*, *Hybridoma 13*: 469 (1994).

Alternatively, the fragment encoding the Δ$CH2h3IgG_1$ constant region can be subcloned into the high-level expression vector, hMN14pdHL2 (Immunomedics, Inc.; Morris Plains, NJ). See Figure 6. This vector contains both heavy and light chain sequences. The vector also includes a dihydrofolate reductase gene to facilitate subsequent amplification with increasing concentrations of methotrexate. The IgG_1 constant region fragment containing CH1, IgG_1 hinge, CH2 and CH3 is removed from the vector by *Hind*III/*Xmn*I digestion. *Hind*III cleaves at the intron sequence joining the constant region to the heavy chain variable domain, while *Xmn*I cleaves at the coding sequences of the CH3 domain. Subsequently, the *Hind*III/*Xmn*I DNA fragment from ΔC2h3pBSK is inserted into the corresponding site of the hMN14pdHL2 construct. The resultant construct, hMN-14ΔC2h3pdHL2 (Figure 7), when transfected into SP2/0 cells, or other mammalian cell lines, will express a hMN14 antibody with the CH2 domain deleted and carrying an IgG_3 hinge region instead of an IgG_1 hinge region.

As discussed above, it may be advantageous to engineer an N-linked glycosylation site for conjugation of additional haptens. The method for introducing a VK-appended glycosylation site has been described by Leung *et al.*, *J. Immunol. 154*: 5919 (1995), and by Hansen *et al.*, U.S. Patent No. 5,443,953 (1995), which are incorporated by reference. Briefly, the mutation is introduced by PCR using a long oligonucleotide (about a 65-mer) that has an identical sequence to the 5' region of the light chain sequence of interest, except at amino acid position 18 - 20 (according to Kabat's numbering system) where the sequence for carbohydrate addition is engineered, and a short 3'-end oligonucleotide with a sequence identical to the C-terminus of the antibody of interest. For example, a vector containing DNA that encodes a mutant light chain, when co-transfected with the heavy chain vector hMN14ΔC2h3pG1g into SP2/0 cells, will express a CEA-specific antibody carrying an IgG_3 hinge region with the CH2 domain deleted, and a light chain variable region glycosylated at amino acid position 18 - 20.

Example 2

Preparation of Immunoconjugates Comprising Drugs, Peptides, and Polymers

A. General Procedures for Preparation of Immunoconjugates

During experimental procedures, reaction conditions can be varied within certain parameters to manipulate the nature of the final product. For example, a mutated antibody is first reduced with 1 - 500 mM solutions of thiols to reduce the desired number of hinge-region disulfide bonds. Thiols such as cysteine, cysteamine, glutathione, mercaptoethanol, mercaptoethylamine, dithiothreitol and dithioerythritol may be used in the practice of the invention. A thiol-containing mutated antibody is advantageously further substituted within 24 hours of preparation, although storage at low temperature

(4°C or frozen) under an argon atmosphere may be employed to stabilize the reduced construct prior to further reactions.

The thiol-containing construct can be compounded for technetium-99m radiolabeling with or without stannous ion. If stannous ion is compounded with the reduced construct, the construct can be labeled with pertechnetate taken directly from a Tc-99m/Mo-99 generator. Alternatively, the mutated antibody can be compounded without the stannous ion and radiolabeled with reduced Tc-99m-tartrate and the like. Technetium-99m labeling of mutated antibodies can be carried out at specific activities up to 150 mCi per milligram of protein. Most preferably, specific activities of 20 - 40 mCi/mg protein are employed. Tc-99m labeling is carried out most usefully in volumes of about 0.5 - 2 milliliters.

B. Conjugation of Drugs

Two classes of antitumor drugs that have been conjugated to Mabs most frequently are anthracyclines and methotrexate. Doxorubicin or cyanomorpholino anthracycline is reacted with bromoacetic acid hydrazide to yield the bromoacetyl hydrazone of the drug. That is, doxorubicin is activated for thiol reaction while an acid-labile hydrazone group has been placed within the linkage. The second generation, more toxic, antitumor agent, cyanomorpholinyl anthracycline is attached to the construct in the same manner.

This aspect of the invention describes the attachment of hydrazone-drug conjugates to free thiol groups of a mutated antibody in a site-specific manner. For analyses, the purity of the immunoconjugates are evaluated initially by size-exclusion HPLC using a Zorbax-250 or Bio-Sil sizing column and appropriate molecular weight standards. Dual wavelength detection at 280 nm for antibody detection and at 495 nm for drug detection are used. Samples are often re-analyzed using buffers with increasing percentages (up to 20%) of

organic buffers to discern if any non-covalently bound drug is associated with the Mab. Ion-exchange columns also are used as a secondary system for the same purpose. Instant thin-layer chromatography (ITLC) systems are used to separate free drug from drug-Mab conjugates. Polyacrylamide gel electrophoresis (PAGE), both native and denatured, under reducing or non-reducing conditions, is used to verify the chromatography results.

Immunoprecipitation is used to determine immunoreactivity retention of the drug conjugates. For immunoprecipitation, radiolabeled immunoconjugates are incubated with rabbit anti-mouse IgG for 1 hour at 4°C. Immunoprecipitin (Gibco BRL; Gaithersburg, MD) is added and, after an additional incubation, the mixture is centrifuged and the pellet separated. The supernatant is treated with 3 N HCl to hydrolyze any drug-Mab conjugate and the aqueous solution neutralized and extracted with an organic solvent. The amount of doxorubicin is quantified by UV spectroscopy at 495 nm, and compared with the amount of radioactivity located in the pellet or in the supernatant to gauge the percentage of non-immunoreactive conjugate.

1. **Conjugation of cyanomorpholinyl anthracycline-2-bromoacetic acid hydrazone to hIMMU-14-IgG_3**

The hIMMU-14-ΔCH2-IgG_3 construct (10 μmol) is reduced with 2-mercaptoethanol (150 mM) at pH 8.7 for 10 minutes at 4°C. To purify the product, the reduction mixture is applied to a spin-column of Sephadex G-50-80 in 0.1 M sodium phosphate (pH 7.5). The number of free sulfhydryl groups in the effluent is determined by the Ellman reaction, and protein concentration is determined by absorbance at 280 nm. See, for example, Coligan at page 9.4.5.

The reduced antibody (10 μmol) in phosphate-buffered 0.9% sodium chloride (pH 7.5) is treated with cyanomorpholino anthracycline-2-bromoacetic acid hydrazone (400 μmol) at 25°C. Residual free thiol groups are tested during the course of the reaction by Ellman

testing of reaction aliquots at hourly time intervals. The conjugation reactions proceeds for about six hours before the Ellman reaction is negative. The cyanomorpholinylanthracycline-hydrazonyl-2-acetamido-S-hIMMU-14-ΔCH2-IgG_3 conjugate is purified by size-exclusion chromatography and the substitution ratio of the drug onto the protein is determined spectrophotometrically, as described above. Approximately 5 - 15 drug molecules are bound to the mutated antibody framework.

2. Conjugation of cyanomorpholinyl anthracycline-2-(bromoacetyl)-6-aminohexanoic acid hydrazone to hIMMU-14-IgG_3

The hIMMU-14-ΔCH2-IgG_3 construct (10 μmol) is reduced with 2-mercaptoethanol (150 mM) at pH 8.7 for 10 minutes at 4°C. To purify the product, the reduction mixture is applied to a spin-column of Sephadex G-50-80 in 0.1 M sodium phosphate (pH 7.5). The number of free sulfhydryl groups in the effluent is determined by the Ellman reaction, and protein concentration is determined by absorbance at 280 nm.

The reduced antibody (10 μmol) in phosphate-buffered 0.9% sodium chloride (pH 7.5) is treated with cyanomorpholino anthracycline, 2-(bromoacetyl)-6-aminohexanoic acid hydrazone (400 μmol) at 25°C. Residual free thiol groups are tested during the course of the reaction by Ellman testing of reaction aliquots at hourly time intervals. The conjugation reactions proceeds for about six hours before the Ellman reaction is negative. The cyanomorpholinyl anthracycline-hydrazonyl-hexanoyl-6-acetamido-S-hIMMU-14-ΔCH2-IgG_3 conjugate is purified by size-exclusion chromatography and the substitution ratio of the drug onto the protein is determined spectrophotometrically, as described above. Approximately 5 - 15 drug molecules are bound to the mutated antibody framework.

3. Preparation of alkylating drug conjugates of the hIMMU-14-ΔCH2-IgG_3 construct

The hIMMU-14-ΔCH2-IgG_3 construct is solubilized in 0.2 M Tris buffer (pH 8.7) at a concentration of 5 - 20 mg/ml, and treated with 2-mercaptoethanol in the same buffer to a final concentration of 1 - 100 mM of the free thiol. After a 10 minute incubation at 4°C, the reduced construct is purified by Sephadex G-50-80 size-exclusion chromatography developed in 0.2 M PBS (pH 7.4 - 8.5) that contains 10 mM EDTA to preserve thiol groups in the reduced state. The reduced mutated construct is then treated with a 10 - 1000 fold molar excess of a triaziridine chemotherapeutic alkylating agent, Trenimon, for 0.15 - 3 hours with stirring. Purified drug-hIMMU-14-ΔCH2-IgG_3 conjugate is obtained by size-exclusion chromatography and stored at pH 4 - 5 to maintain the stability of the appended chloroethyl groups. The number of reactable chloroethyl groups per mutated antibody is determined by duplicate/triplicate reaction with known amounts of low molecular weight thiol, followed by estimation of residual unreacted thiol using a procedure such as the Ellman reaction. Remaining thiol is determined by measuring absorbance at 410 nm, and the level of chlorambucil substitution is determined by subtracting this number from the total amount of free thiol added.

4. Substitution of an hIMMU-14-ΔCH2-IgG$_3$ construct with multiple chloroethyl groups

Thiol-reduced hIMMU-14-ΔCH2-IgG$_3$ construct is solubilized in 0.1 M phosphate-buffered saline (pH 7.4 - 8.5) at a temperature of 4 - 25°C. The construct is incubated for 1 - 5 hours with stirring in 10 - 15% dimethylsulfoxide (DMSO) with 10 - 50-fold molar excess (to free thiols on the construct) of a bis-alkylating agent such as sulfur or nitrogen mustard dissolved in aqueous DMSO. Chloroethyl-substituted hIMMU-14-ΔCH2-IgG$_3$ construct is purified by size-exclusion chromatography, and stored in 0.1 M acetate buffered saline (pH 5.0). The number of chloroethyl groups per antibody moiety is determined as described above.

C. Conjugation of Polypeptides

1. Preparation of streptavidin-hIMMU-14-ΔCH2-IgG$_3$

Streptavidin is activated for specific thiol coupling to mutated antibody by reaction with the amino-thio cross-linker sulfosuccinimidyl-4-[N-maleimidomethyl]cyclohexane-1-carboxylate (sulfo-SMCC; Pierce Chemical Co., Rockford, IL). A streptavidin solution (2.9 ml) containing 29 mg of streptavidin dissolved in 0.1 M phosphate buffered saline (PBS), pH 7.3, is treated with 2.42 ml of a freshly prepared solution of 10 mM sulfo-SMCC in water. After one hour of stirring at room temperature, the protein is purified from low molecular weight materials on a Sephadex G-50-80 size-exclusion column run in 0.1 M PBS (pH 7.0). The maleimido-streptavidin is reconcentrated to about 5 to 6 ml for reaction with reduced mutated antibody.

A solution of 12 mg of 2-mercaptoethylamine in 53 μl of 0.1 M ethylenediaminetetraacetic acid (EDTA) and one milliliter of 0.1 M PBS (pH 7.3) is freshly prepared. Six hundred microliters of this solution is added to 2.5 ml of an 18 mg/ml solution of the mutated antibody in 0.1 M PBS (pH 7.3). After a 45 minute incubation at room temperature, the reduced construct is purified by size exclusion chromatography on a Sephadex G-50-80 column run

in 0.1 M PBS (pH 7). The product is concentrated to 3 - 4 ml for the conjugation reaction.

Each of the maleimido-streptavidin and the reduced mutated antibody is added in six aliquots to 8 ml of 0.1 M PBS (pH 7) with rapid stirring over a period of 5 to 6 minutes. Stirring is continued for one hour at room temperature. Then, 25 mg of sodium tetrathionate are added to the mixture to block unreacted maleimides and stirring is continued for another five minutes. The product, 1:1 mutated antibody-streptavidin, is purified from aggregated and unreacted proteins and low molecular weight materials by preparative size-exclusion chromatography on a 600 x 21.5 mm TSK-SW-3000 column run at 1.5 ml/min in 0.2 M PBS (pH 6.8).

2. Substitution of cysteinyl-peptide haptens onto an hIMMU-14-ΔCH2-IgG$_3$ construct

The hIMMU-14-ΔCH2-IgG$_3$ construct is solubilized in 0.2 M Tris buffer (pH 8.7) at a concentration of 5 - 20 mg/ml, and treated with 2-mercaptoethanol in the same buffer to a final concentration of 1 - 100 mM of the free thiol. After a 10 minute incubation at 4°C, the reduced construct is purified by Sephadex G-50-80 size-exclusion chromatography developed in 0.2 M PBS (pH 7.4 - 8.5).

The reduced mutated construct is then treated with an excess of 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent). The modified construct is purified by size-exclusion chromatography in 0.1 M borate buffer (pH 8), and treated with an excess of cysteinyl peptide (C-ttp2). The progress of the disulfide exchange reaction is monitored by the production of the 3-carboxylato-4-nitrothiophenolate anion.

D. Conjugation of Polyethylene Glycol

A mutated antibody (15 mg/ml) is reduced with a solution of 20 mM cysteine and 2 mM EDTA in 40 mM PBS (pH 6.6) for 60 minutes at 37°C. The reduced mutated antibody is purified on two 3 ml spin columns that contain Sephadex G-50-80 equilibrated in 0.1 M PBS (pH 7.0) that contains 2 mM EDTA. The purified construct is

then treated with a 10-fold molar excess of polyethylene (PEG)-maleimide (Shearwater Polymers, Inc.; Huntsville, AL) in the same buffer. After a one hour incubation at room temperature, the mutated antibody-PEG conjugate is purified by size-exclusion chromatography to provide a product containing 5-8 PEG polymers per mole of construct.

Example 3

Preparation of Radiolabeled Immunoconjugates

A. Radiolabeling Mutated Antibodies using Bifunctional Chelates

1. Attachment of a bifunctional chelate to mutated antibody and radiolabeling with a metal nuclide

Mutated antibody is reduced with 2-mercaptoethanol (25 mM) at pH 8.7 for 10 minutes at 4°C. For purification, the reduction mixture is applied to a spin column of Sephadex G-50-80 in 0.1 M sodium phosphate (pH 8.1). The number of sulfhydryl groups in the effluent is determined by the Ellman reaction.

A mixture of reduced mutated antibody (7.3 mg) and 8μl of 2-(p-bromoacetamido)benzyl-DTPA (Br-Bz-DTPA) (0.23 μmole) is incubated at 37°C for 30 minutes at pH 8.1. The conjugate is purified on a one milliliter spin column of Sephadex G-50-80 in 50/150 mM acetate/sodium chloride (pH 5.3). The conjugate concentration of the effluent is determined by absorbance at 280 nm to be 13.3 mg/ml. The chelate/antibody ratio is determined by metal binding assay.

$^{111}InCl_3$ is purchased from a supplier such as New England Nuclear. It is diluted with 0.1 M ammonium citrate (pH 5.58) to give a solution of 15 μCi/μl. This mixture is incubated for one hour at room temperature for 30 minutes prior to use.

An aliquot of the conjugate (5μl, 0.43 nmol) is mixed with 5 μl of carrier-added indium citrate (4.9 nmol) and 10 μl of 0.1 M ammonium citrate (pH 5.58), and incubated at room temperature. ITLC run in 10 mM EDTA shows that

36% of the indium is bound to the conjugate. To remove nonspecifically bound indium, a one microliter aliquot is mixed with a solution of 1 μl of 0.1 M EDTA and eight microliters of water, and the mixture is incubated for ten minutes. ITLC in 10 mM EDTA (pH 6.4) shows 18% of the indium bound to the conjugate. Accordingly, the chelate/antibody ratio is calculated to be 2.

To radiolabel for imaging purposes, mutated antibody-Bz-DTPA (104 μl, 1.5 mg) is treated with 280 μl of ^{111}In (5.4 mCi) in 0.1 M ammonium citrate. The radiolabeling mixture is incubated at room temperature for one hour. Nonspecifically bound indium is removed by treating the reaction mixture with EDTA (final concentration: 10mM) for ten minutes at room temperature. ITLC in 10 mM EDTA shows 84% of the radioactivity bound to antibody. The mutated antibody-Bz-DTPA-^{111}In is purified on a one milliliter spin column of Sephadex G-50-80, 50/150 mM acetate/sodium chloride (pH 5.3). HPLC of the effluent shows the product eluting at 8.6 minutes as a single peak. To ensure complete removal of unbound ^{111}In, the EDTA chase with ITLC is repeated on an aliquot of the purified radioimmunoconjugate. ITLC shows 99% of the radioactivity bound to protein, while immunoreactivity measurement on a CEA-affigel column is measured at 90%.

Mutated antibody can be labeled with yttrium-90 as follows. A solution of mutated antibody-S-Bz-DTPA (60 μl, 1 mg) in 0.1 M sodium acetate buffer (pH 6.5) is added to a solution of yttrium-90 (0.5 ml, 5mCi) in 0.5 M sodium acetate buffer (pH 6). The solution is mixed and incubated at room temperature for one hour. After this time, the Y-90-mutated antibody is diluted to 5 ml using 2% human serum albumin in 0.1 M PBS (pH 7), containing 1 mM EDTA. After 15 minutes, ITLC of an aliquot of the diluted labeling mixture, run in 0.01 M EDTA shows 96% of the radioactivity associated with the protein construct.

2. Attachment of a radiolabeled bifunctional chelate to mutated antibody

As an alternative, a bifunctional chelate can be labeled with a radioactive isotope, and the radiolabeled bifunctional chelate is then conjugated with a mutated antibody. For example, the activated bifunctional chelate, 2-p-(bromoacetamido)benzyl-diethylenetriaminepentaacetic acid (Br-Bz-DTPA) (5 μl, 145 nmol) is mixed with Indium-111 citrate (5μl, 60μCi) and ammonium citrate (5μl, 0.1 M, pH 5.85). The reactants are incubated at room temperature for 15 minutes. ITLC shows quantitative binding of the indium radionuclide by Br-Bz-DTPA. Reduced mutated antibody (0.5 ml, 16.6 mg/ml), prepared as described above, in 50 mM HEPES buffer (pH 8.3) is mixed with ^{111}In-Br-Bz-DTPA (1.5 μl, 14.6 nmol of Br-Bz-DTPA). The protein:DTPA ratio is about 1:1, although the SH:Br reactant ratio is greater than 5:1. The mixture is incubated at 37°C and the progress of the reaction is monitored by size-exclusion HPLC using an in-line radiation detector. HPLC analysis shows 50%, 85% and 100% incorporation of radioactivity into mutated antibody at 30 minutes, one hour and two hours post-incubation, respectively.

B. Direct Labeling with Radionuclides

1. Tc-99m-hIMMU-14-ΔCH2-IgG$_3$

The hIMMU-14-ΔCH2-IgG$_3$ (10 μmol), constructed as described above, is reduced with 2-mercaptoethanol (150 mM) at pH 8.7 for 10 minutes at 4°C. For purification, the reduction mixture is applied to a spin column of Sephadex G-50-80 in 0.1 M sodium acetate (pH 5.5) that contains 0.9% sodium chloride (ABS buffer). The number of free sulfhydryl groups in the effluent is determined to be 20 per mole of protein by the Ellman reaction. Protein concentration is determined by measuring absorbance at 280 nm. The reduced mutated antibody is aliquoted into 100 μg - 2 mg fractions and stored under argon frozen (or at 4°C) prior to radiolabeling.

Alternatively, the reduced mutated antibody is compounded with stannous ion and lyophilized for radiolabeling in the future. In the latter procedure, each 200 μg of the hIMMU-14-ΔCH2-IgG_3-SH construct (5-10 SH/mole protein) is mixed with a solution of 25 μg of stannous chloride in 150 μl of 50 mM ABS buffer containing sodium potassium tartrate (9.2 mM) and lyophilized in a two milliliter glass vial. The lyophilizate is stored under partial vacuum/argon or under full vacuum in a septum-sealed vial.

Tc-99m radiolabeling is performed by adding 5 mCi (1 ml) of Tc-99m pertechnetate generator-eluate to achieve a specific activity of 25 mCi/mg. The labeling may be performed on the freshly formulated material without the lyophilization step. Radio-HPLC analysis at 30 minutes post-labeling shows 95-100% incorporation of radioactivity into the antibody construct.

2. Re-188-hIMMU-14-ΔCH2-IgG_3

A solution of stannous chloride dihydrate in 6N HCl at a stannous ion concentration of 100 mg/ml together with a solution of 100 mM sodium potassium tartrate (a stannous ion complexor) in 50 mM sodium acetate buffer (pH 5.3) and a solution of 1 M sodium acetate buffer (pH 6) to neutralize the excess hydrochloric acid are used. For each vial containing 1 mg of the hIMMU-14-ΔCH2-IgG_3-SH construct to be radiolabeled with rhenium-188, 480 μl of the tartrate solution is treated with 6.6 μl of the Sn(II)/HCl solution (equivalent to 660 μg of stannous ion) and mixed briefly by vortex. This mixture is added to the one milligram of the construct and the entire mixture is lyophilized.

Rhenium-188 is conveniently obtained from a tungsten-188/rhenium-188 generator system such as the one developed at Oak Ridge National Laboratory. Re-188-perrhenate is eluted with sterile physiological saline to provide the isotope (up to a Curie) in a 5 to 20 milliliter volume of solution. The vial containing the hIMMU-14-ΔCH2-IgG_3-SH is reconstituted with Re-188

perrhenate, mixed to ensure dissolution and allowed to stand for two hours at room temperature. The radiolabeled product is analyzed by HPLC on a Bio-Sil 250 size-exclusion column (Bio-Rad; Hercules, CA) fitted with an in-line radioactivity detector, and run at 1 ml/minute in 0.2 M phosphate buffer (pH 6.8). ITLC on a silica gel impregnated glass fiber strips (Gellman Sciences; Ann Arbor, MI) activated according to the manufacturer's instructions also are used for analysis. These analyses show greater than 90% incorporation of the Re-188 isotope into the hIMMU-14-ΔCH2-IgG_3-SH construct.

3. Ag-111-hIMMU-14-ΔCH2-IgG_3

hIMMU-14-ΔCH2-IgG_3 (1 ml, 15 mg/ml) in 0.18 M Tris-HCl buffer (pH 8.1) at 4°C is treated with 2 μl of 2-mercaptoethanol, mixed thoroughly, and incubated for 10 minutes. Excess 2-mercaptoethanol is removed on a Sephadex G-50-80 size exclusion column in ABS buffer (pH 4.5). The effluent fraction containing protein is measured for protein concentration by absorbance at 280 nm. The concentration of thiol groups generated is determined by reaction with Ellman's reagent. A ratio of 15 - 20 sulfhydryl groups per antibody is found. Reduced antibody construct is aliquoted in 1 mg portions into 2 ml vials, optionally lyophilized, and stored under partial vacuum/argon to preserve the thiol groups for Ag-111 radiolabeling.

Silver-111 nitrate is diluted with water to 5 mCi/ml. A labeling volume of 1 ml is used to reconstitute (if necessary) a sample of lyophilized hIMMU-14-ΔCH2-IgG_3-SH (at pH 5) in 50 - 100 mM ABS buffer (1 mg). After thorough mixing, the solution is incubated at room temperature for 30 minutes, and the Ag-111 labeled conjugate is then tested. Instant thin-layer chromatography on silica gel impregnated glass strips with two developing systems are used: one system to separate unbound silver from protein-bound silver (10 mM EDTA) and the other system to separate all soluble forms of silver from precipitated silver (5:2:1 water:

ethanol:concentrated ammonium hydroxide). Less than 1% insoluble silver-111 is seen in the labeling mixture. HPLC on size-exclusion columns is used to confirm ITLC data, with HPLC recovery quantitated. Greater than 95% of the applied Ag-111-hIMMU-14-ΔCH2-IgG_3 is recovered from the HPLC column.

Although the foregoing refers to particular preferred embodiments, it will be understood that the present invention is not so limited. It will occur to those of ordinary skill in the art that various modifications may be made to the disclosed embodiments and that such modifications are intended to be within the scope of the present invention, which is defined by the following claims.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those in the art to which the invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference in its entirety.

What Is Claimed Is:

1. A mutated antibody comprising a heavy chain having (a) a variable region, (b) a CH1 domain, (c) a hinge region having three or more disulfide bonds, and (d) a CH3 domain, and lacking a CH2 domain.

2. The mutated antibody of claim 1, further comprising a light chain that comprises a carbohydrate moiety attached at about amino acid 18 in the variable region of said light chain.

3. The mutated antibody of claim 2, further comprising an agent selected from the group consisting of calicheamicin, esperamicin and polyethyleneglycol, wherein said agent is attached to said mutated antibody by said carbohydrate moiety.

4. The mutated antibody of claim 1, wherein said hinge region is a human IgG_3 hinge region.

5. The mutated antibody of claim 4, wherein said variable region binds with an antigen that is associated with a tumor or an infectious agent.

6. An immunoconjugate comprising the mutated antibody of claim 5 and at least one diagnostic or therapeutic agent.

7. The immunoconjugate of claim 6, wherein said diagnostic agent is selected from the group consisting of radioactive label, photoactive agent or dye, florescent label, enzyme label, bioluminescent label, chemiluminescent label, colloidal gold and paramagnetic ion.

8. The immunoconjugate of claim 7, wherein said radioactive label is selected from the group consisting of γ-emitters and positron-emitters.

9. The immunoconjugate of claim 8, wherein said γ-emitters have a gamma radiation emission peak in the range of 50-500 Kev.

10. The immunoconjugate of claim 9, wherein said γ-emitters with a gamma radiation emission peak in the range of 50-500 Kev are selected from th group consisting of ^{99m}Tc, ^{67}Ga, ^{111}In, ^{123}I, ^{125}I and ^{131}I.

11. The immunoconjugate of claim 6, wherein said therapeutic agent is selected from the group consisting of radioisotope, boron addend, immunomodulator, toxin, photoactive agent or dye, cancer chemotherapeutic drug, antiviral drug, antifungal drug, antibacterial drug, antiprotozoal drug and chemosensitizing agent.

12. The immunoconjugate of claim 11, wherein said radioisotope is selected from the group consisting of α-emitters, β-emitters, γ-emitters, Auger electron emitters, neutron capturing agents that emit α-particles and radioisotopes that decay by electron capture.

13. The immunoconjugate of claim 1, wherein said radioisotope is selected from the group consisting of ^{198}Au, ^{32}P, ^{125}I, ^{131}I, ^{90}Y, ^{186}Re, ^{188}Re, ^{67}Cu and ^{211}At.

14. A composition for treating a mammal having either a tumor that expresses a tumor associated antigen or a disease caused by an infectious agent, wherein said composition comprises an immunoconjugate comprising:

(a) a mutated antibody comprising a heavy chain having (i) a variable region that binds with an antigen associated with a tumor or an infectious agent, (ii) a CH1 domain, (iii) a hinge region having three or more disulfide bonds, and (iv) a CH3 domain, and lacking a CH2 domain, and

(b) at least one therapeutic agent.

15. The composition of claim 14, wherein said therapeutic agent is selected from the group consisting of radioisotope, boron addend, toxin, immunomodulator, photoactive agent or dye, cancer chemotherapeutic drug, antiviral drug, antifungal drug, antibacterial drug, antiprotozoal drug and a chemosensitizing agent.

16. The composition of claim 15, wherein said radioisotope is selected from the group consisting of α-emitters, ß-emitters, γ-emitters, Auger electron emitters, neutron capturing agents that emit α-particles and radioisotopes that decay by electron capture.

17. The composition of claim 16, wherein said radioisotope is selected from the group consisting of ^{198}Au, ^{32}P, ^{125}I, ^{131}I, ^{90}Y, ^{186}Re, ^{188}Re, ^{67}Cu and ^{211}At.

18. A composition for detecting the location of antigen associated with tumor cells or antigen associated with an infectious agent in a mammal having either a tumor that expresses a tumor associated antigen or a disease caused by an infectious agent, wherein said composition comprises an immunoconjugate comprising:

(a) a mutated antibody comprising a heavy chain having (i) a variable region that binds with an antigen associated with a tumor or an infectious agent, (ii) a CH1 domain, (iii) a hinge region having three or more disulfide bonds, and (iv) a CH3 domain, and lacking a CH2 domain, and

(b) at least one diagnostic agent.

19. The composition of claim 18, wherein said diagnostic agent is selected from the group consisting of radioactive label, photoactive agent and paramagnetic ion.

20. The composition of claim 19, wherein said radioactive label is selected from the group consisting of γ-emitters and positron-emitters.

21. The composition of claim 20, wherein said γ-emitters have a gamma radiation emission peak in the range of 50-500 Kev.

22. The composition of claim 21, wherein said γ-emitters with a gamma radiation emission peak in the range of 50-500 Kev are selected from the group consisting of ^{99m}Tc, ^{67}Ga, ^{123}I, ^{125}I and ^{131}I.

23. A method for detecting the presence of antigen associated with tumor cells or antigen associated with an infectious agent in a biological sample, said method comprising the steps of:

(a) contacting said biological sample with an immunoconjugate comprising:

(i) a mutated antibody that comprises a heavy chain having (1) a variable region that binds with

an antigen that is associated with a tumor or an infectious agent, (2) a CH1 domain, (3) a hinge region having three or more disulfide bonds, and (4) a CH3 domain, and lacking a CH2 domain, and

(ii) a detectable marker.

(b) detecting any of said immunoconjugate bound to said biological sample.

24. The method of claim 23, wherein said detectable marker is selected from the group consisting of radioactive label, fluorescent label, enzyme label, bioluminescent label, chemiluminescent label and colloidal gold.

25. The method of claim 24, wherein said radioactive label is selected from the group consisting of ^{3}H, ^{125}I, ^{131}I, ^{35}S and ^{14}C.

26. The method of claim 24, wherein said fluorescent label is selected from the group consisting of fluorescein isothiocyanate, rhodamine, phycoerytherin, phycocyanin, allophycocyanin, *o*-phthaldehyde and fluorescamine.

27. The method of claim 24, wherein said enzyme label is selected from the group consisting of β-galactosidase, glucose oxidase, peroxidase and alkaline phosphatase.

28. The method of claim 24, wherein said bioluminescent label is selected from the group consisting of luciferin, luciferase and aequorin.

29. The method of claim 24, wherein said chemiluminescent label is selected from the group consisting of luminol, isoluminol, an aromatic acridinium ester, an imidazole, an acridinium salt and an oxalate ester.

FIG. 1

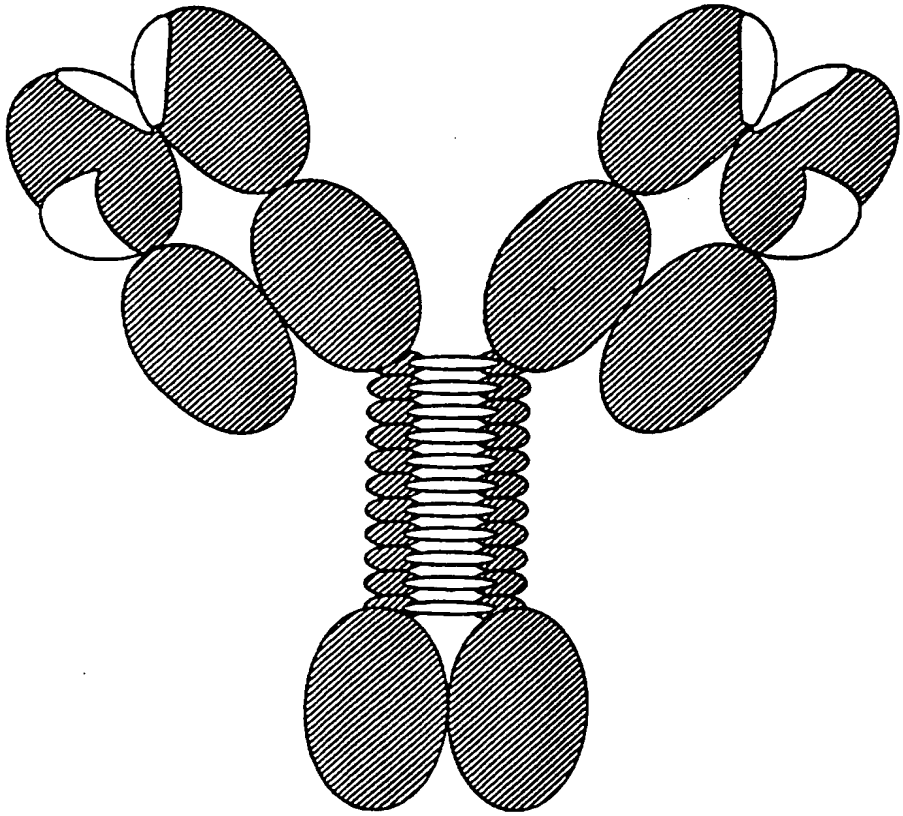
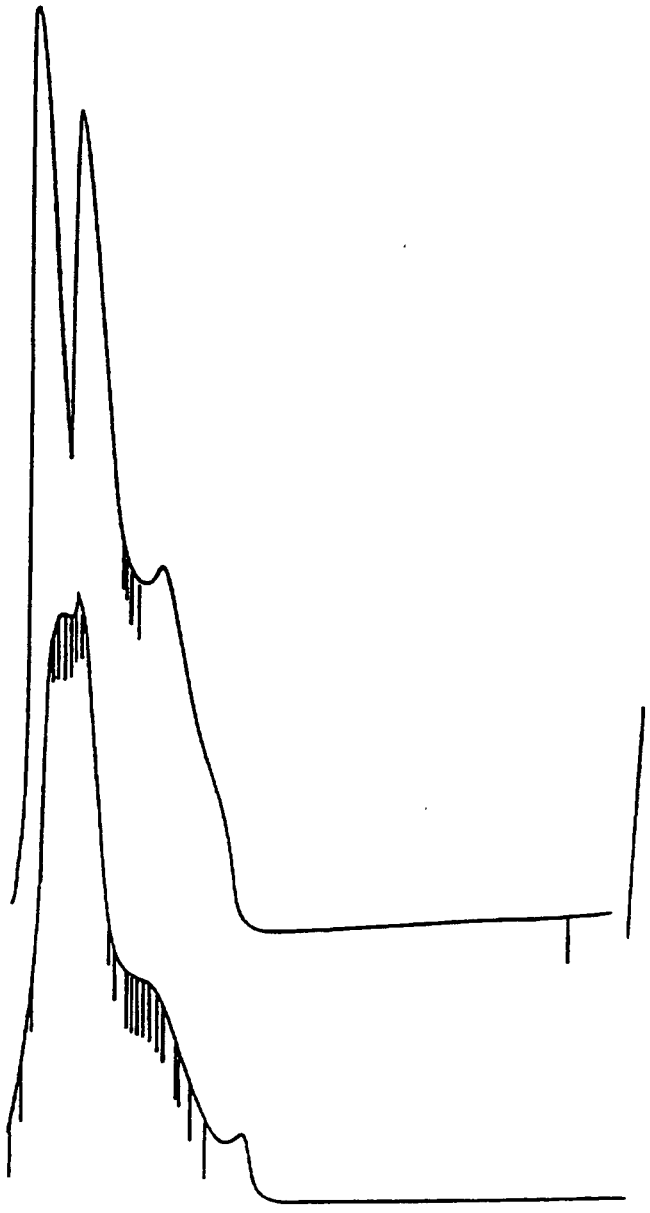


FIG. 2



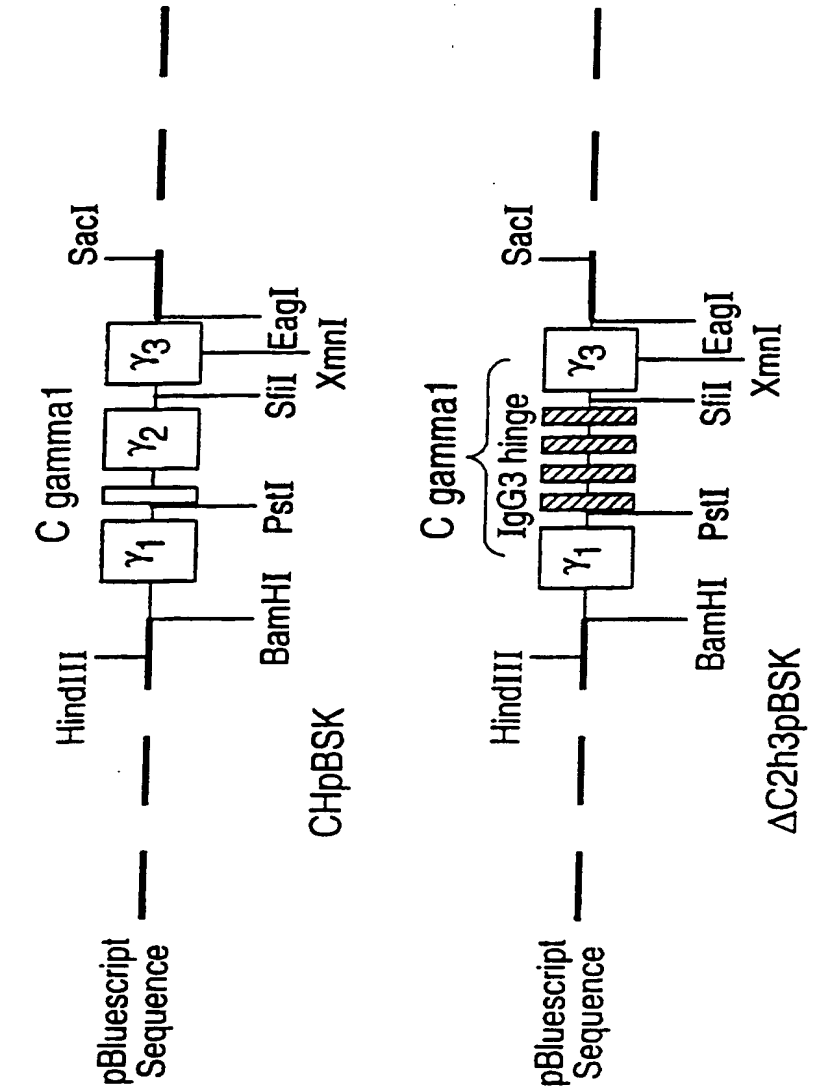

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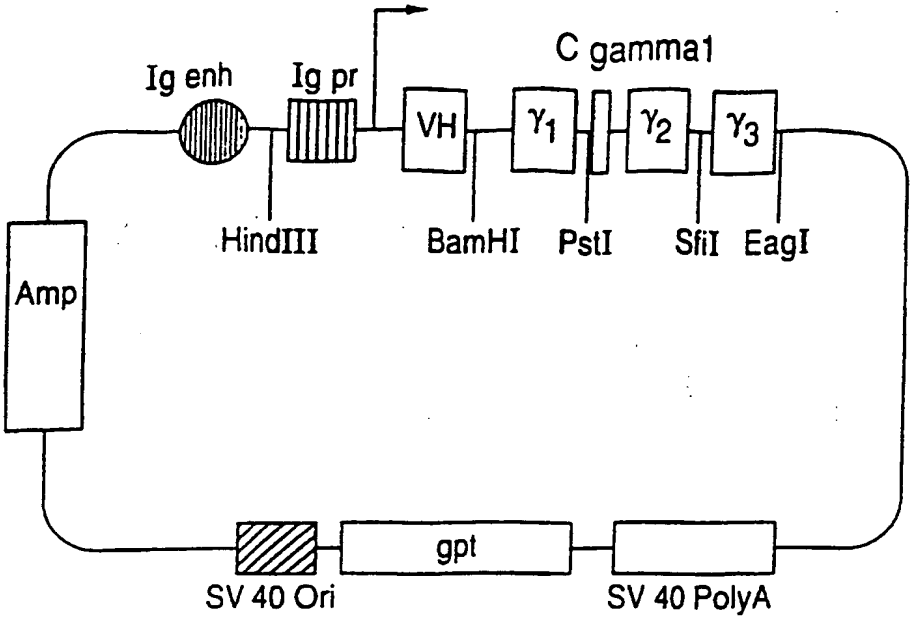
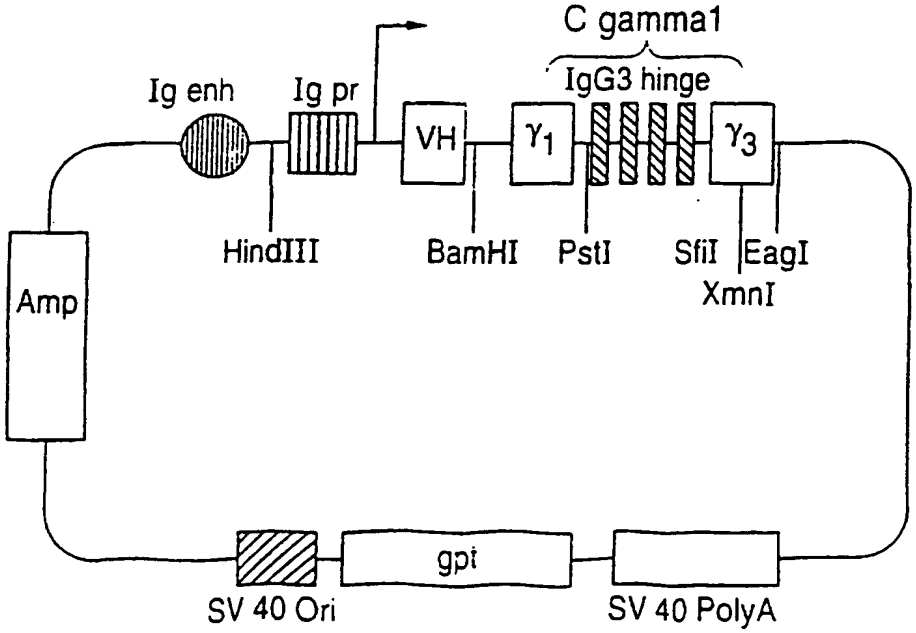
FIG. 4

FIG. 3



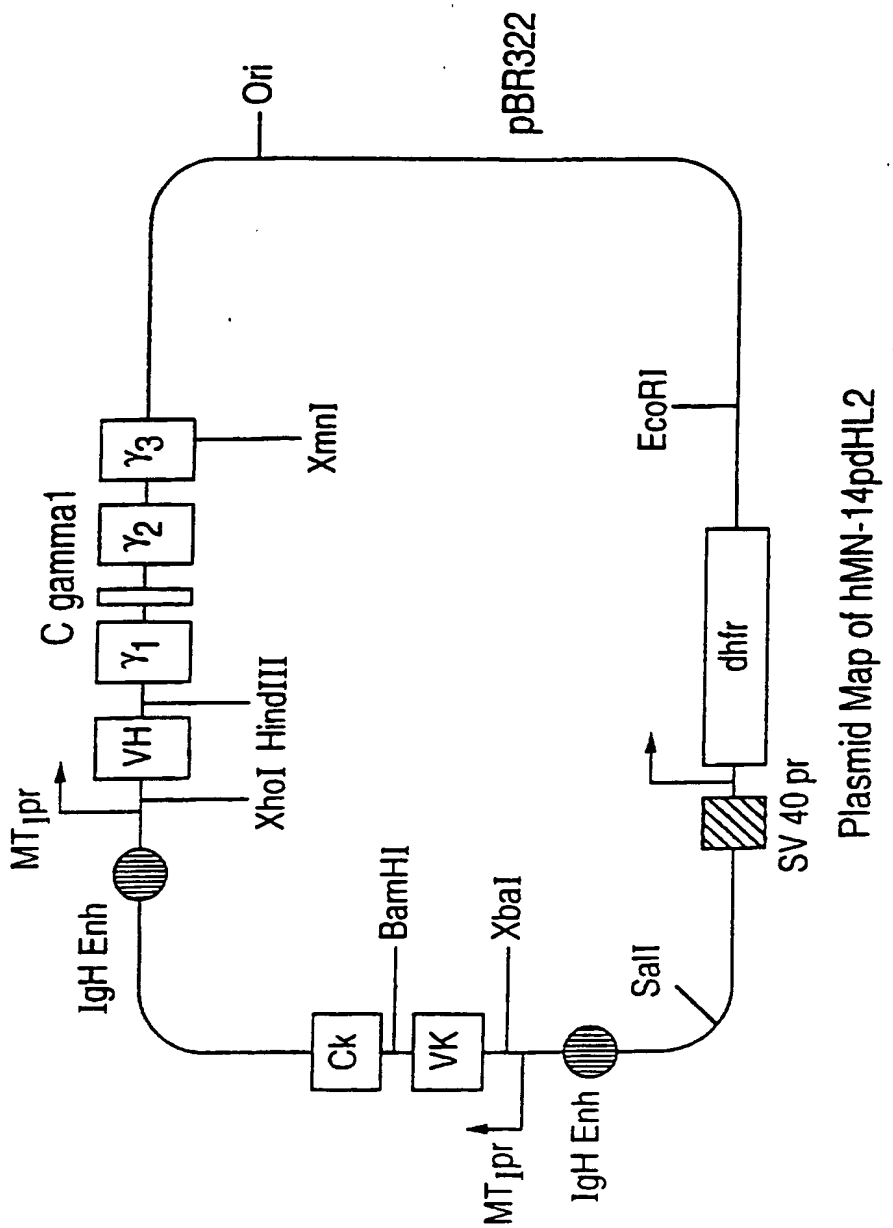
Plasmid Map of LL2-14pG1g

FIG. 5



Plasmid Map of hMN-14ΔC2h3pG1g

FIG. 6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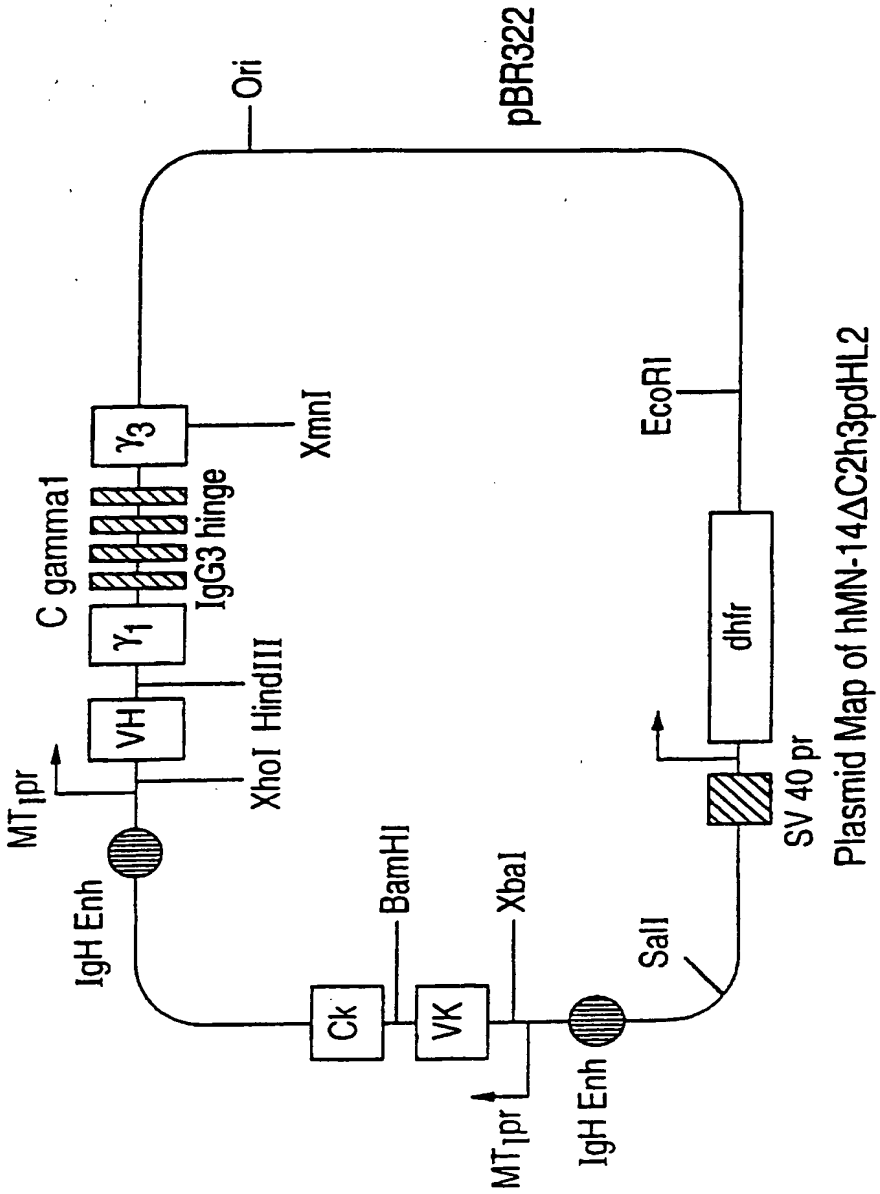
Plasmid Map of hMN-14pdHL2

FIG. 7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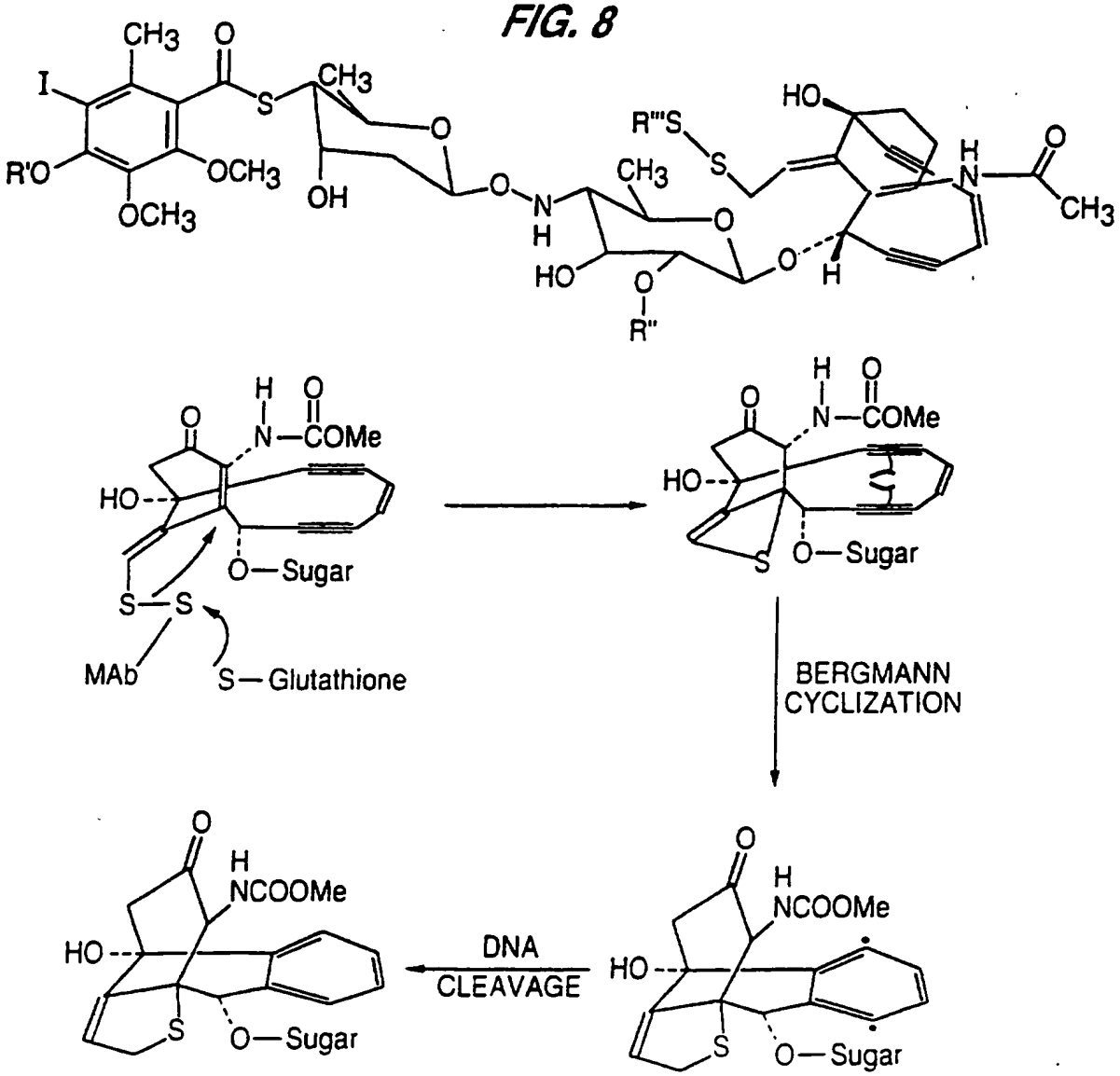
Plasmid Map of hMN-14ΔC2h3pdHL2

FIG. 8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INTERNATIONAL SEARCH REPORT

Inten. .onal application No.
PCT/US96/14832

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 33/53; A61K 39/395; C07K 16/00
US CL :424/9.1, 133.1, 179.1; 435/7.23; 530/387.3, 391.5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/9.1, 133.1, 179.1; 435/7.23; 530/387.3, 391.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS
search terms: mutated antibody, chimeric, CH1 domain, variable region, carbohydrate attachment

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 95/15769 A1 (IMMUNOMEDICS, INC.) 15 June 1995, see entire document.	1-29
X	LEUNG et al. Engineering a unique glycosylation site for site-specific conjugation of haptens to antibody fragments. Journal of Immunology. 1995, Vol. 154, No. 11, pages 5919-5926, especially page 5919.	1-29
X	LEUNG et al. Grafting a unique carbohydrate-addition-site for site-specific conjugations: 1. Molecular biology and immunochemistry. Journal of Nuclear Medicine. May 1994, Vol. 35, No. 5, supplement. Abstract No. 243. See Abstract.	1-29

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
25 NOVEMBER 1996

Date of mailing of the international search report
24 DEC 1996

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
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Authorized officer
JULIE REEVES

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

2/3/1

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XRAM Acc No: C99-180531
XRPX Acc No: N99-455755
Method to treat alcoholism named a triple defence

Patent Assignee: MEDVEDEV V M (MEDV-I)
Inventor: MEDVEDEV V M; PONOMAREVA A G
Number of Countries: 001 Number of Patents: 001
Patent Family:

Patent No	Kind	Date	Applicat No	Kind	Date	Main IPC	Week
RU 2113241	C1	19980620	RU 9445662	A	19941229	A61M-021/00	199953 B

Priority Applications (No Type Date): RU 9445662 A 19941229

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011234688
WPI Acc No: 97-212591/199719
XRAM Acc No: C97-068616
XRPX Acc No: N97-175365
Mutated antibodies having a hinge region with disulphide bonds and lacking a CH2 domain - form immuno-complexes with diagnostic or therapeutic agents and for delivery to specific target areas e.g. tumours

Patent Assignee: IMMUNOMEDICS INC (IMMU-N)
Inventor: GRIFFITHS G L; LEUNG S
Number of Countries: 074 Number of Patents: 005
Patent Family:

Patent No	Kind	Date	Applicat No	Kind	Date	Main IPC	Week
WO 9711370	A1	19970327	WO 96US14832	A	19960920	G01N-033/53	199719 B
JP 11514223	W	19991207	WO 96US14832	A	19960920	C12N-015/09	200008
			JP 97512815	A	19960920		
AU 9671604	A	19970409	AU 9671604	A	19960920	G01N-033/53	199731
EP 861440	A1	19980902	EP 96933032	A	19960920	G01N-033/53	199839
			WO 96US14832	A	19960920		
AU 702975	B	19990311	AU 9671604	A	19960920	G01N-033/53	199922

Priority Applications (No Type Date): US 954169 A 19950922
Filing Details:

Patent	Kind	Filing Notes	Application	Patent
WO 9711370	A1			
		Designated States (National): AL AM AT AU AZ BB BG BR BY CA CH CN CU CZ DE DK EE ES FI GB GE HU IL IS JP KE KG KP KR KZ LC LK LR LS LT LU LV MD MG MK MN MW MX NO NZ PL PT RO RU SD SE SG SI SK TJ TM TR TT UA UG US UZ VN		
		Designated States (Regional): AT BE CH DE DK EA ES FI FR GB GR IE IT KE LS LU MC MW NL OA PT SD SE SZ UG		
JP 11514223	W	Based on		WO 9711370
AU 9671604	A	Based on		WO 9711370
EP 861440	A1	Based on		WO 9711370

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010690812 **Image available**
WPI Acc No: 96-187768/199619
XRAM Acc No: C96-059971
New intermediates for prepn. of esperamicin - which is useful as an antibiotic
Patent Assignee: SCRIPPS RES INST (SCRI)
Inventor: CLARK D A; NICOLAOU K C
Number of Countries: 001 Number of Patents: 001
Patent Family:

Patent No	Kind	Date	Applicat No	Kind	Date	Main IPC	Week
US 5504206	A	19960402	US 94312106	A	19940926	C07D-265/12	199619 B

Priority Applications (No Type Date): US 94312106 A 19940926
Language, Pages: US 5504206 (7)

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010551260
WPI Acc No: 96-048213/199605
XRAM Acc No: C96-016418
New cytotoxic drug conjugates having very high selectivity for target cells - prepd. from methyltrisulphide antitumour antibiotics and new linker system
Patent Assignee: AMERICAN CYANAMID CO (AMCY)
Inventor: HALLETT W; HAMANN P R; HINMAN L; HOLCOMB R; HOLLANDER I; TSOU H; WEISS M J
Number of Countries: 024 Number of Patents: 016
Patent Family:

Patent No	Kind	Date	Applicat No	Kind	Date	Main IPC	Week
NO 9502206	A	19951204	NO 952206	A	19950602	A61K-047/48	199605 B
AU 9520449	A	19951214	AU 9520449	A	19950602	C07H-015/203	199606
EP 689845	A2	19960103	EP 95303746	A	19950601	A61K-047/48	199607
FI 9502720	A	19951204	FI 952720	A	19950602	A61K-000/00	199609
CA 2150785	A	19951204	CA 2150785	A	19950601	C07K-016/00	199614
JP 8048637	A	19960220	JP 95155644	A	19950531	A61K-039/44	199617
ZA 9504570	A	19960731	ZA 954570	A	19950602	A61K-000/00	199635
NZ 272274	A	19971124	NZ 272274	A	19950602	C07K-014/71	199802
US 5739116	A	19980414	US 94253877	A	19940603	A01N-043/04	199822
			US 95461284	A	19950605		
BR 1100990	A3	19980519	BR 971100990	A	19970514	A61K-039/39	199826
BR 1100941	A3	19980526	BR 971100941	A	19970514	C07K-016/22	199827
US 5767285	A	19980616	US 94253877	A	19940603	C07D-207/46	199831
			US 95462939	A	19950605		
US 5773001	A	19980630	US 94253877	A	19940603	A61K-039/395	199833
BR 1101078	A3	19980922	BR 971101078	A	19970514	C07C-063/00	199845
AU 697280	B	19981001	AU 9520449	A	19950602	C07H-015/203	199851
US 5877296	A	19990302	US 94253877	A	19940603	C07K-016/00	199916
			US 95452164	A	19950526	T	